PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	Rajagopalan et al.
Serial No.	10/808,184
Filing Date	March 24, 2004
Art Unit	1612
Confirmation No.	4580
Examiner	Packard, Benjamin J
Title	NOVEL AROMATIC AZIDES FOR TYPE I PHOTOTHERAPY
Attorney Docket No.	1486.1 H US (073979.68)

Cincinnati, OH 45202 October 10, 2008

DECLARATION OF JOHN K. BUOLAMWINI, Ph.D.
PURSUANT TO 37 C.F.R. §1.132

I, John K. Buolamwini, declare as follows:

I am a Medicinal Chemist and hold the rank of Full Professor in the Department of Pharmaceutical Sciences at the University of Tennessee Health Science Center, College of Pharmacy. My Curriculum Vitae is attached. I have read the specification of U.S. Patent Application Serial No. 10/808,184 as it was filed with the U.S. Patent and Trademark Office, the claims currently pending, and the June 23, 2008 Office Action.

I understand that the Examiner holds the opinion that the specification does not disclose sufficient information so that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention at the time the application was filed, which is referred to as the "written description" requirement in the Office Action. I understand that a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventors had possession of the claimed invention. I understand that possession can be shown with words, structures, figures, diagrams, and structural chemical formulas. I understand that actual reduction to practice is not required.

The instant application claims a method of performing a phototherapeutic procedure in which the azide, as part of the formula

$$E - L - Ar - X - N_3$$

with E, L, Ar, and X defined as recited in claim 11, is administered to a patient, allowed to accumulated in target tissue, and then the target tissue is exposed to light at a wavelength between 300 and 1200 nm with sufficient power and fluence rate to perform the procedure.

I understand that the Examiner's Action limits E = bombesin receptor binding molecule, L = $(CH_2)_a$, Ar = benzene, and X = single bond. Thus, the limited formula can be indicated as

$$\text{bombesin receptor binding molecule} - (CH_2)_a - Ar - N_3$$

Based on the documents that I reviewed, I was asked if and how I could determine each of the following issues:

- a "bombesin receptor binding molecule" description
- whether and where a "receptor binding molecules" would attach to a methene group (with methene defined as R-CH=R')
- whether the receptor binding molecule must have a peptide chain, and how a receptor binding molecule that does not have a peptide backbone would bind.

I understand that the Examiner finds the application does not describe the above issues. The Examiner states at pp. 2-3 "The description requirement of the patent statute requires a description of an invention, not an indication of a result that one might achieve if one made that invention". I respectfully disagree with this assertion. It is my opinion that, based on the application as filed, I am able to address each of the above issues, as I subsequently explain. It is my opinion that putting the invention together and using it as described requires a level of experimentation that is reasonable for one skilled in this art; it is not "undue".

I know, and assert that a person of ordinary skill in the art would know, or could determine without undue experimentation, the structure of the disclosed receptor binding molecules, e.g., a bombesin receptor binding molecule, the example cited by the Examiner. There is a correlation between structure and function in a molecule that is a ligand to a receptor. It is precisely because of the structure that the molecule binds to the receptor; the binding establishes a structure/function relationship. In reciting bombesin receptor binding molecules, one is specifically defining a structure that binds the bombesin receptor, which one skilled in the art can quickly identify knowledge or performing a literature search. Molecules fitting the "receptor binding" claim terms are those known to bind the receptor at the time of invention. More specifically, at the time of the invention there were definite numbers of known or reported ligands or binding molecules for all the receptors as the claims recite. Thus in so stating, the Applicants are not just outlining goals that they hope the claimed invention would achieve, but

give clear description to one skilled in the art as to what molecule to use as the targeting group. This is not indefinite, as the literature at the time reveals all such reported molecules.

I now describe bombesin receptor binding molecules, and attach all the references to which I cite.

Bombesin constitutes a family of peptides involved in mitogenesis. "Bombesin receptor binding molecules" include agonists and antagonists for the bombesin receptor. Agonists include, e.g., the endogenous ligands gastrin-releasing peptide (GRP) (Oiry et al., A synthetic glycine-extended bombesin analogue interacts with the GRP/bombesin receptor, Eur. J. Pharmacol. 403 (2000) 17; Heizmann et al., A combinatorial peptoid library for the identification of novel NSH and GRP/bombesin receptor ligands, Recept Signal Transduct Res. 19 (1999) 449; Varvarigou et al., Synthesis, Chemical, Radiochemical and Radiobiological Evaluation of a New ^{99m}Tc-labelled Bombesin-like Peptide, Cancer Biotherapy & Radiopharmaceuticals, 17 (2002) 317; Nagy et al., Design, synthesis, and in vitro evaluation of cytotoxic analogs of bombesin-like peptides containing doxorubicin or its intensely potent derivative, 2-pyrrolinodoxorubicin, Proc. Natl. Acad. Sci, USA 94 (1997) 652); neuromedin B (NMB), GRP-18-27, and [D-Cys^{6},Cys^{13}, Leu^{14}]Bn(6-14)(XIII) (Coy et al., Covalently Cyclized Agonist and Antagonist Analogues of Bombesin and Related Peptides, J. Biol. Chem., 266 (1991) 16441). Antagonists include, e.g., [Leu^{13}-ψ-CH$_2$NH-Leu^{14}]bombesin (Coy et al., Probing Peptide Backbone Function in Bombesin, J. Biol. Chem. 263 (1988) 5056); JMV-1458 (glycine-extended bombesin (paraphydroxy-phenyl-propionyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-OH), JMV-641, JMV-1799, and JMV-1802 (each in Oiry et al., A synthetic glycine-extended bombesin analogue interacts with the GRP/bombesin receptor, Eur. J. Pharmacol. 403 (2000) 17); PD165929, 1-naphthoyl-[DAla^{24},DPro^{26},ψ26-27]GRP-20-27, kuwanon H, and kuwanon G. Conjugation of bombesin receptor binding peptides to other compounds is known in the art, such as the conjugation of the anticancer drug doxorubicin or its analog, as described by Nagy et al., Proc. Natl. Acad. Sci. 94 (1997) 652.

A bombesin receptor binding molecule that does not have a peptide backbone could be derivatized to contain a peptide by which it could bind using standard conjugation techniques know to a person of ordinary skill in the art. Such a derivatization has been published (see Zhou et al., Clin. Cancer Res. 9 (2003) 4953).

I therefore assert that a person of ordinary skill in the art would know, or could determine without undue experimentation, the structure that meets the stated function, would understand the term "receptor binding molecule", and would understand the structural requirements based on

the general binding functionality language. Thus, the use of "receptor binding molecules" is not indistinct.

I assert that the structure of the targeting group was sufficiently definite at the time of the invention. As a medicinal chemist who makes molecules that bind primarily to receptors or enzymes, I cannot immediately profane a molecule that binds to a receptor unless I have seen that molecule described as a ligand for the receptor, or I myself have made such a molecule. In the former case I can propose a potential ligand that will be a derivative or analog of an already known molecule. That does not mean that the molecule does not exist, however, and it does not mean that I cannot, by a single literature search, uncover it. It is reasonable that a chemist or medicinal chemist will perform a literature search to find a molecule that will bind a receptor. I assert that a bombesin receptor binding molecule is an art-recognized structural term. When one hears these as a medicinal chemist, one can envision such molecules. For example, E could be an antibody or part of a monoclonal antibody–FAB fragment, there are methods for linking antibodies to other compounds, etc. (see Zhou et al., Clin. Cancer Res. 9 (2003) 4953).

The Examiner states "Further, it is unclear that the receptor binding molecule must have a peptide chain. In that case, the declaration does not overcome the ability to bind a receptor binding molecule which does not have a peptide backbone" (top p. 5). It is clear to me that there is no requirement that the receptor binding molecule must have a peptide chain. It is clear to individuals skilled in the art that receptor binding molecules may have a peptide chain or may not. The disclosure does not require that the receptor binding molecule have a peptide chain. As long as a molecule (i.e., E) can be conjugated to the Ar moiety, it is suitable.

The azide is a photoreactive compound that will undergo photolysis upon irradiation with electromagnetic radiation to expel nitrogen gas and produce nitrene species that can destroy tissues or cells. The choice of E will depend on the receptor to which E should bind, which in turn is dictated by the disease to be targeted.

Knowing this, I would perform a literature search to find molecules (ligands) reported to bind to the chosen receptor (those particular receptor binding molecules). Once I have chosen the desired molecule based on its high affinity for the receptor, I would perform a chemical reaction to link the molecule to the dye with a linker, also linking the dye to the azide.

Upon performing my literature search, I would identify molecules with high affinity binding, preferably having low nanomolar K_d values, and molecules that have a structure such that they can be conjugated to the dye which has activatable carboxylic acid groups. For

example, I would identify compounds having a primary amino group that is not sterically hindered, and use this group to link to the Ar-X-azide through L. If such a compound is not available, I would engineer a handle with a spacer on the ligand molecule at the appropriate substitution position. This might take some time to do. It may be that I have to perform screening assays to discover such a compound and use it, which is quite feasible.

To carry out the claimed method, I would decide for which disease to use phototreatment. I would then determine which receptors are highly or selectively expressed in that tissue or, if cancer, the cancer cells. I would then select a targeting molecule or ligand to that receptor (i.e. E) then link E to the aryl azide. Companies sell such linking agents. Moreover, as already stated, conjugation of bombesin receptor binding peptides to other compounds is known in the art, such as the conjugation of the anticancer drug doxorubicin or its analog, as describe by Nagy et al. (Proc. Natl. Acad. Sci. 94 (1997) 652). With regard to attachment to a methene, the presence of an alkyl halide moiety on the Ar is one example that should allow a nucleophilic displacement reaction with a suitably situated nucleophile such as NH_2, SH, or OH on the biomolecule to effect the attachment. Such nucleophilic substitution is a standard reaction in synthetic organic chemistry.

After linking E to the aryl portion, which is linked to the azide, I would purify it, then assay binding with the receptor preferably expressed on the tissues or cells I intend to destroy in the photo procedure. I would use a known binding molecule (ligand) to the receptor as a competitive ligand to see how well it is displaced, or its binding is inhibited, by the E-L-aryl azide conjugate. From this experiment, I would determine the binding affinity in the form of a K_d value. The competitive ligand could be the molecule E that was coupled to the dye. The attachment of E to the aryl azide might affect the binding affinity of E to the receptor, and this experiment would show whether or not E still binds strongly enough to the receptor to make the product useful for the photo procedure. Thus, it is my opinion that one skilled in the art would immediately recognize competitive assay as one type of binding assay.

Once satisfied that the product binds sufficiently to the receptor, I would make a pharmaceutical formulation of the compound, apply it to the tissues at the doses indicated, wait for the appropriate time to allow the compound to bind to the tissues or cells, and then irradiate the tissues, for example, with a fiber optic tube using a laser at the specified wavelengths. Other considerations may be toxicity of the product to the host or patient, which would be determined by the stability of the azide as well as the targeting group or ligand, and may not be a concern.

Locating the claimed formula at the receptor so that the azide can be photoactivated is required; the inventors are not claiming any particular binding property. The binding assays will be performed under physiological conditions of buffer pH and temperature etc. In my opinion, (1) any binding that sufficiently locates the azides at the claimed receptors will suffice; and (2) one skilled in the art knows or can readily determine without undue experimentation which compounds will locate the azide at the claimed receptors, as I have previously described.

It is my opinion that the state of the art is well developed to make the claimed compounds and perform the claimed methods. The descriptions can be understood by anyone skilled in the art of medicinal chemistry. Ordinary chemists and medicinal chemists would be able to put a composite molecule together, and pharmaceutical scientists would be able to formulate the compound for administration. The experimentation is involved with the literature search to identify E to attach to the aryl azide, determining binding affinity, and optimizing spacer length to enhance binding affinity. It is my opinion that such experimentation is routine for a medicinal chemist and is not undue experimentation. Further, there are bombesin receptor binding kits to evaluate bombesin receptor binding compounds (e.g., DELFIA Bombesin Receptor Binding Kit, PerkinElmer (Boston MA)).

I respectfully assert that there is a correlation between the structure of the claimed formula and its function. The specification discloses that the azide group is key to the photoactivating process. Nitrenes are produced by azides upon exposure to light of the proper wavelength. E is the group that binds to one of the claimed receptors and hence locates the formula at the desired site.

Based on the teachings of the specification, I understand that E would serve to locate the active portion of the molecule to the "target" site to be treated. This target is a cell or tissue containing, in this case, a bombesin receptor. Thus, a compound that binds to bombesin receptors would locate the azide to the desired site. As described, the results of my literature search would allow me to envision the molecules that would bind to the bombesin receptor. The selection, addition, and evaluation of such a targeting compound is not, in my opinion, "undue" experimentation because the identity, availability, affinity, avidity, testing, etc. of such receptor binding compounds are established in the art. In my opinion, any experimentation to formulate or enhance such targeting would certainly not be "undue", but instead would be encompassed by routine organic synthesis and/or receptor binding assays as I have described.

For at least the reasons I have set forth, I respectfully assert that one skilled in the art would be able to make the claimed invention without undue experimentation, and that the inventors were in possession of their invention at the time they filed the instant application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the subject application or any patent issued thereon.

10/10/2008
Date

John K. Buolamwini, Ph.D.

CURRICULUM VITAE

NAME: **John K. Buolamwini** Address: 1809 Oak Springs Drive, Cordova, TN 38016

EDUCATION:

Undergraduate: University of Science and Technology, Ghana, 1977-1981, **B. Pharm. Honors** (1981).
Graduate/ University of Alberta, Edmonton, Canada, 1985-1990, **Ph.D**. (1990) Medicinal Chemistry.
Professional: Registrant of the Pharmacy Examining Board of Canada (1993)

Postdoctoral:

1990-1992: Alberta Cancer Board Fellow, University of Alberta, Canada,
1992-1994: NIH Fogarty Fellow, National Cancer Institute, NIH, Bethesda, MD,

UNIVERSITY APPOINTMENTS:

07/05-Present: Professor of Pharmaceutical Sciences
Department of Pharmaceutical Sciences
College of Pharmacy,
University of Tennessee Health Science Center, Memphis, TN

10/04-Present: Associate Program Leader of Developmental Therapeutics
University of Tennessee Cancer Institute
University of Tennessee Health Science Center, Memphis, TN

8/00-6/05: Associate Professor of Pharmaceutical Sciences
Department of Pharmaceutical Sciences
College of Pharmacy,
University of Tennessee Health Science Center, Memphis, TN

7/00-8/00: Associate Professor of Pharmaceutical Sciences
Department of Medicinal Chemistry, and
Research Associate Professor of the Research Institute of Pharmaceutical Sciences,
School of Pharmacy,
University of Mississippi, Oxford, MS

9/94 to 6/00: Assistant Professor of Pharmaceutical Sciences
Department of Medicinal Chemistry, and
Research Assistant Professor of the Research Institute of Pharmaceutical Sciences,
School of Pharmacy, University of Mississippi, Oxford, MS

OTHER PROFESSIONAL ACTIVITIES/HONORS/AWARDS:

2008	Clinical and Experimental Therapeutics-1 (CET-1) peer review panel of the 2008 Department of Defense Breast Cancer Research Program (BCRP)
2005-2008	National Cancer Institute Drug Discovery/Development Program Project (PO1) Special Emphasis Study Sections
2007-2008	Member, NIH Cancer Drug Development and Therapeutics SBIR/STTR ZRG1 ONC-V Special Emphasis Study Sections.
2007	Member, Translational Research Review Panel, Ontario Institute of Cancer Research, Canada
2005	Member, Program Committee, American Association for Cancer Research (AACR) 96th Annual National Meeting, 2005
2004	Chair, Minisymposium on Experimental Therapeutics, American Association for Cancer Research (AACR) 95th National Meeting, Orland, FL
2003-Present	Member, ZRG1 AARR-E (16): NIAID, NIH AIDS Drug Discovery SBIR/STTR Study Section
2002- Present	External Grant Reviewer, Florida A & M University NIH MBRS & RCMI Grants Programs
2002	Ad Hoc Reviewer, National Science Foundation (NSF, Chemistry Section)
2000-Present	Editor-in-Chief, *Current Cancer Drug Targets*
2000	Executive Guest Editor, *Current Pharmaceutical Design* (Novel Cancer Molecular Targets Issue)
2000-2005	NIH KO1 Research Career Development Award
1998-2000	Legislative Committee Member of the American Association for Cancer Research
1996-1997	New Investigator Award, American Association of Colleges of Pharmacy (AACP)
1996-1998	Young Investigator Award, American Society of Pharmacognosy
1996 & 2002	Grant Reviewer, American Chemical Society Research Fund
1995-Present:	Rho Chi National Pharmacy Honor Society
1992-1994	Fogarty International Fellowship Award, National Institutes of Health, Bethesda, MD
1985-1990	Ph.D. Studentship Award, Alberta Heritage Foundation for Medical Research, University of Alberta, Canada

SOCIETY MEMBERSHIPS:

American Chemical Society
American Association for Cancer Research,
American Association of Colleges of Pharmacy

INVITED LECTURES/SEMINARS

Division of Basic Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Florida A. & M. University, Tallahassee, Florida. "*Workshop on Molecular Modeling for Drug Discovery*". July 27, 2007. (Invited Lecturer)

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL. *Computer-Aided Drug Design*, June 17, 2007 (Visiting Lecturer).

Nelson Institute of Environmental Sciences, New York University School of Medicine, Tuxedo, NY. "*Nucleoside Transporters as Therapeutic Targets: Inhibitors and Probes*". September 29, 2005 (Invited Speaker)

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL. *Computer-Aided Drug Design*, January 18, 2005 (Visiting Lecturer).

World Pharmaceutical Congress, Cheminformatics Track, Cambridge Healthtech Institute, Philadelphia, PA. "QSAR in Lead Development" May 24-25, 2005 (Invited Speaker)

Department of Pharmacology, University of North Carolina-Chapel Hill, Chapel Hill, NC. "*Nucleoside Transporters as Therapeutic Targets: Inhibitors and Probes*". September 28, 2004 (Invited Speaker)

Eli Lilly Pharmaceutical Company, Computational Chemistry Department, Indianappolis, Indiana "*QSAR and Docking Approaches to Exploring Drug Binding Modes and Receptor Selectivity*". June 25, 2004. (Invited Speaker)

Department of Biochemistry, Mehary Medical College, Nashville, TN. "*Application of Computer-Aided Molecular Design to Cancer Drug Discovery: p53-MDM2 Interaction, GSTPI, and Receptor Tyrosine Kinase Inhibitors*". December 15, 2003 (Invited Speaker)

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL. *Computer-Aided Drug Design*, January 27, 2003 (Visiting Lecturer).

Department of Chemistry, Tennessee Technical University, Cookeville, TN, *Approaches to the Design and Discovery of Novel Agents Against Molecular Targets in Heart Disease, Stroke, Cancer and AIDS.* February 20, 2001 (Invited Speaker)

Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, TN, *Approaches to the Design and Discovery of Novel Agents Against Molecular Targets in Heart Disease, Stroke, Cancer and AIDS.* May 8, 2000 (Invited Speaker)

Delta State University, Cleveland, MS, Mississippi Alliance for Minority Participation Guest Speaker, November 15, 1999 (Invited Speaker)

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL. "*Molecular Modeling Approaches to the Design and Discovery of Inhibitors of Nucleoside Transporters or Glutathione S-Transferase Pi Polymorphic Enzymes*". June 2, 1999. (Invited Speaker)

Department of Chemistry, University of Arkansas at Pine Bluff, Arkansas. "*Computer-Aided Ligand Design and Molecular Biology Approaches to the Discovery of Novel Agents for the Treatment of Heart Disease, Stroke and Cancer*". March 4, 1999. (Invited Speaker)

Division of Basic Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Florida A. & M. University, Tallahassee, Florida. "*Computer-Aided Ligand Design and Molecular Biology Approaches to the Discovery of Novel Agents for the Treatment of Heart Disease, Stroke and Cancer* and *AIDS*". July 16, 1998. (Invited Speaker)

Department of Pharmaceutical Chemistry, College of Pharmacy, Rutgers University, Piscataway, NJ, *Application of Computer-Aided Ligand Design and Molecular Biology Approaches to the Discovery of Novel Agents for the Treatment of Heart Disease, Stroke and Cancer* June 25, 1998. (Invited Speaker)

Department of Medicinal Chemistry, School of Pharmacy, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA. "*Application of Computer-Aided Ligand Design and Molecular Biology Approaches to the Discovery of Novel Agents for the Treatment of Heart Disease, Stroke and Cancer".* February 18, 1998 (Invited Speaker)

Department of Chemistry, University of Memphis, Memphis, TN. "*Computer-Aided Design and Discovery of Nucleoside Transport Inhibitors*". October 20, 1995 (Invited Speaker)

Department of Medicinal Chemistry, University of Mississippi, Oxford, U.S.A. "*An Integrated Approach to the Discovery of Molecular Targets and Agents for the Therapy of Cancer and AIDS*". March 4, 1994 (Invited Speaker)

Department of Physiology, University of Alberta, Edmonton, Canada. "*Structure-Function Relationships of SAENTA-fluorescein Conjugates: Molecular Modeling Studies*" February 8, 1994 (Invited Speaker)

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada. "*An Integrated Approach To The Discovery of New Molecular Targets And Agents For The Therapy of Cancer and AIDS*" February 15, 1994 (Invited Speaker)

Departments of Chemistry and Biochemistry, University of Saskachewan, Saskatoon, Canada. "*Molecular Modeling Studies of Structure-Property Relationships of SAENTA-fluorescein Conjugates:* " February 22, 1994 (Invited Speaker)

Department of Pharmacology, University of Alberta, Edmonton, Canada "*Structure-Function Relationships of SAENTA-fluorescein Conjugates: Molecular Studies*" February 28, 1994 (Invited Speaker)

EDITORIAL APPOINTMENTS:

2000 to Present Editor-in-Chief, *Current Cancer Drug Targets* Bentham Science Publishers (http://www.bentham.org/ccdt)
Executive Guest Editor *Current Pharmaceutical Design* Issue on "Novel Molecular Targets for Anticancer Drug Discovery" (February 2000)
Editorial Board Member of *Current Medicinal Chemistry* (1997 to Present)
Editorial Board Member of *Medicinal Chemistry*, (2004 to Present)

PEER REVIEWER OF MANUSCRIPTS FOR THE FOLLOWING JOURNALS:
Journal of Medicinal Chemistry
European Journal of Medicinal Chemistry
Bioorganic and Medicinal Chemistry
Bioorganic and Medicinal Chemistry Letters
Journal of Natural Products

Expert Opinion in Investigational Drugs
Expert Opinion in Therapeutic Patents
Journal of Pharmacy and Pharmaceutical Sciences
Nature Reviews Cancer
Cancer Research
Clinical Cancer Research
Molecular Cancer Therapeutics
Journal of the National Cancer Institute
Cancer Detection and Prevention
Molecular Pharmacology
Journal of Computer-Aided Molecular Design
Journal of Biomolecular Screening,
FEBS Letters
Pharmacology Biochemistry and Behavior.
Drug Discovery Today

COMMITTEES AND OFFICES

2000-Present	University of Tennessee Health Sciences Center Institutional Review Board (IRB)
2008-	University of Tennessee College of Pharmacy Scholarships and Awards Committee
2008-	University of Tennessee Health Sciences Center Faculty Senate
2006-2007	University of Tennessee Health Sciences Center Strategic Planning Committee, Implementation
2004-2007	University of Tennessee College of Pharmacy Research Committee
2003-Present	University of Tennessee College of Pharmacy Tenure & Promotion Committee
2002	University of Tennessee College of Pharmacy Financial Resources Committee
1998-2000	Chancellor's Task Force on Academic Computing, University of Mississippi
1996-1997	Library Council, University of Mississippi
1998-2000	Coordinator, Year 2000 (Y2K) Computer Compliance for Dept. of Med. Chem.
1996-2000	Member, Computer Committee, School of Pharmacy, University of Mississippi,
1999-2000	Member, Science Library Self-Study Committee, University of Mississippi,
1998-2000	Member, Animal Welfare Committee, School of Pharmacy, University of Mississippi
1998-2000	Member Greenhouse and Garden Committee, School of Pharmacy, University of Mississippi
1998-2000	Member Scholastic Standards Committee, School of Pharmacy, University of Mississippi

NATIONAL INSTTITUTES OF HEALTH (NIH) EXTRAMURAL GRANT FUNDING

RO1 CA112519 (NIH/NCI) (PI: Ali-Osman) 02/1/06-01/31/11

Novel Targeted Therapeutics for CNS Malignancies

The long-term objective of this project includes structure-based design, molecular modeling and synthesis and lead optimization of novel compounds as GST-pi enzyme-targeted anti-glioma therapeutics.
Role: Co-PI (PI for UT Subcontract for structure-based design, molecular modeling and chemical synthesis)

R03 AI077478 (NIAID/NIH) (PI: Buolamwini) 02/15/08-1/31/010

Carcinogenicity Testing of Novel Phenanthrene Diketoacid Anti-HIV Agents

The goal of this project is to undertake structure-activity relationship (SAR) studies and test the carcinogenicity of novel phenanthrene diketoacids.
Role: Principal Investigator

R21 AI-065372 (NIH/NIAID) **(PI: Buolamwini)** **4/15/2005-3/31/2007**
Nucleoside Transporters In HAART Mitochondrial Toxicity
The goal of this project is to develop a novel nucleoside transport inhibitory approach to preventing mitochondrial toxicity of HAART chemotherapy in HIV/AIDS patients.
Role: Principal Investigator

R03 CA125850 (NIH/NCI) **(PI: Buolamwini)** **07/01/07-05/31/09**
Development of Novel Chemopreventive Agents
The goal of this project is to conduct structure-activity relationship (SAR) studies for the discovery of novel chemopreventive agents
Role: Principal Investigator

P50CA108786 (NIH/NCI SPORE) **(PI: Ali-Osman)** **09/01/04-8/31/09**
Development of GSTP1-Targeted Anti-Glioma Therapeutics
The long-term objective of this project is to optimize and develop lead candidates with high in vitro and in vivo antiglioma activity for human clinical trials.
Role: Co-Investigator (PI-UT-Subcontract modeling and synthesis)

PO1 AI057836 (NIH/NIAID) **(PI: McNeil)** **05/01/04-04/30/09**
MDR-TB Drugs: Targeting Cell Wall Synthetic Enzymes
The major goals of this program project are to develop new drugs to treat MDR M. tuberculosis active against cell wall biosynthesis. The project includes structure-based design, medicinal chemistry, testing against essential enzymes, testing against M. tuberculosis bacteria, and testing in mice.
Role: Co-Leader Project 1 (Lee: Project 1 Leader): Structure-based design and QSAR.

R03 CA105327 (NIH/NCI) **(PI: Buolamwini)** **09/22/04-08/31/07**
Nucleoside Transport Inhibitors For Cancer Prevention
The goals of this project are to probe the changes in nucleoside transporter gene expression during carcinogenesis progression, to determine whether nucleoside transport inhibitors can inhibit carcinogenesis.
Role: Principal Investigator

R15 CA-101856 (NIH/NCI) **(PI: Buolamwini)** **7/1/2003-6/31/2006**
Nucleoside Transporters as Chemoprevention Targets
The goal of this project is to investigate nucleoside transporters as chemoprevention targets.
Role: PI

R15 CA-100102 (NIH/NCI) **(PI: Buolamwini)** **06/1/03-05/31/06**
Novel Agents Targeted To p53-Mdm2 Pathways
The goal of this project is to investigate the mechanism of anticancer activity of a series of p53 pathway active compounds discovered through *Structure-Based Drug Design*, exert their effects on p53 and p21 levels by inhibiting p53-Mdm2 interaction, and conduct structure-activity relationship (SAR) studies.
Role: PI

KO1 HL67479 (NIH/NHLBI) **(PI: Buolamwini)** **9/01/2000-8/31/2005**
NBMPR-Binding Site of The Human es Adenosine Transporter.

The long-term objective of this project is to synthesize a novel bifunctional photoaffinity probe and use it to determine amino acids involved in NBMPR binding at the human *es*/ENT1 adenosine transporter.
Role: PI

R01 CA79644 (NIH/NCI) **(PI: Ali-Osman)** **2/01/1999-1/31/2004**
Glutathione S-Transferase pi Polymorphisms and Drug Resistance
The long-term objective of this project is to use molecular modeling and biological studies to understand the mechanisms of interaction of GST-pi variants with substrates and to design new inhibitors of the variant enzymes.
Role: Co-PI

JOURNAL PUBLICATIONS AND BOOK CHAPTERS

1. Buolamwini, J. K. and Knaus, E. E. Synthesis and Antinociceptive Activity of 7-Aryloxysulfonyl Piperidines. *Drug Design and Delivery 3*, 35-47 (1988).
2. Buolamwini, J. K. and Knaus, E. E. Synthesis and Antinociceptive Activity of 4-Pyridyl and -Dihydropyridyl analogues of Meperidine and Ketobemidone. *Drug Design and Delivery 7*, 19-31 (1990).
3. Buolamwini, J. K. and Knaus, E. E. Synthesis and Antinociceptive Activity of 3-Methyl Derivatives of 4-(Pyridyl) Isosteres of Meperidine. *Drug Design and Discovery 8*, 145-156 (1991).
4. Buolamwini, J. K. and Knaus, E. E. Synthesis and Antinociceptive Activity of 1-[2-(pyridyl)ethyl] and 1-[2-(dihydropyridyl)ethyl] Analogues of Fentanyl. *Drug Design Discovery 8*, 307-312 (1992).
5. Buolamwini, J. K. and Knaus, E. E. Synthesis and Analgesic Activity of 3-Methyl Derivatives of 4-(Pyridyl) Isosteres of Ketobemidone. *Eur. J. Med. Chem. 27*, 81-86 (1992).
6. Buolamwini, J. K. and Knaus, E. E. Synthesis and Antinociceptive Activity of 3-Methyl Derivatives of 4-(Pyridyl) Isosteres of Meperidine. *Drug Design Discovery 8*, 245-256 (1992).
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GRADUATE STUDENT DISSERTATION COMMITTEES

Jin Xin, Ph.D. Candidate, Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, 2006-Present

Surekha Pimple, M.S. Thesis Committee Department of Pharmaceutical Sciences, University of Tennessee College of Pharmacy, 2005-2008 (Chairman)

Kris Virga, Ph.D. Candidate, Interdisciplinary Program, University of Tennessee Health Science Center, 2002-2006 (Chairman)

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Wenwei Lin, Ph.D. Candidate, Department of Pharmaceutical Sciences, University of Tennessee College of Pharmacy, 2003-2006 (Chairman)

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Gangadhar Durgam, Ph.D. Candidate, Department of Pharmaceutical Sciences, University of Tennessee College of Pharmacy, 2003-2005

Rajani Kurukonda, Ph.D. Student, Department of Pharmaceutical Sciences, University of Tennessee College of Pharmacy, 2001-2005

Jie Han, Ph.D. Student, Department of Pharmaceutical Sciences, Pharmaceutical Science, University of Tennessee College of Pharmacy, 2001-2003 (Completed August 2003)

Haregewein Assefa, Ph.D. Medicinal Chemistry (University of Mississippi): "*Design and Synthesis of Semi-Synthetic and Partial Analogs of Oleanolic Acid as Potential Complement Inhibitors* " (Completed May, 1999).

James Parker, Ph.D., Chemistry Department (University of Mississippi): *Ab Initio Methods and I.R. Spectroscopy in the Study of Short-lived Chemical Species Using Photolysis and Matrix Isolation.* (Completed July 1999).

Theresa Johnson, Ph.D. Candidate, Medicinal Chemistry (University of Mississippi): *Design and Synthesis of Novel Inhibitors of Lactate Dehydrogenase as Potential Male Contraceptive Agents.* (1997- 2000).

Maria Alvim-Gaston, Ph.D. Candidate, Medicinal Chemistry (University of Mississippi): *Design and Synthesis of Novel Artemisinin Analogs as Antimalarial Agents.* (Completed, June 2000).

John Satumba, Ph.D. Candidate; Chemistry Department (University of Mississippi): "*Folding and Assembly Kinetics of lamda Cro* "(1998-2000).

Community Service

1998-1999	Sigma Xi Graduate Poster Judge, University of Mississippi
1997-1999	Regional Science Fair Judge, Region 7, Mississippi
1998-2000	Oxford Elementary School Student Code of Conduct Committee
1998-2000	Member of Code of Conduct Committee, Oxford City School District, Oxford, MS
2004	Regional Science Fair Judge, Tennessee

FELLOWS/GRADUATE STUDENTS TRAINED:
Postdoctoral Fellows/Research Associates

Name of Fellow: James K. Addo
Degree: Ph.D.
Date Started: November 1, 2000
Date Ended: November 19, 2002
Present Position: Research Scientist (Bioorganic Chemistry), Victoria University of New Zealand.

Name of Fellow: Haregewein Assefa
Degree: Ph.D.
Date Started: February 1, 2001
Date Ended: October 18, 2002
Present Position: Scientist II (Medicinal Chemistry), Paratek Pharmaceuticals Inc., Boston, MA

Name of Fellow: Shantaram Kamath
Degree: Ph.D.
Date Started: April, 2002 to June 2006
Present Position: Pharmacist

Name of Fellow: Shivaputra Patil
Degree: Ph.D.
Date Started: August 03, 2003 to Present
Present Position: Research Associate, Dept. of Pharmaceutical Sciences

Name of Fellow: Chunmei Wang,
Degree: Ph.D.
Date Started: February 2006
Present position: Still a Postdoc in Dr. Buolamwini's Laboratory

Graduate Students Trained:
Name: Amol Gupte
Degree: Ph.D.
Years: August 01-October 06

Name: Wenwei Lin
Degree: Ph.D.
Years: September 01-October 06
Present Position: Postdoctoral Fellow, St. Jude Children's Research Hospital, Memphis, TN

Name: Zhengxiang Zhu
Degree: Ph.D.
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Present Position: Postdoctoral Fellow Columbia University, New York.

Name: Surekha Pimple
Degree: M.Sc.
Years: August 05-March 08
Present Position: Ph.D. Student

Graduate Student Mentoring (Major Advisor)
Joohee Hong a Medicinal Chemistry masters student (1995-1996), pursued a Ph.D. degree in chemistry at the University of California, Santa Barbara, CA.

Maisa Jaradat a Medicinal Chemistry Ph.D. student (1996/97), at the University of Mississippi, who moved into the pharmacology department at the University of Mississippi.

Rodderic Buford a Medicinal Chemistry Masters student (1998-2000) at the University of Mississippi Masters Thesis Title: "Computer-Aided Design and Discovery of Novel HIV Integrase Inhibitors"

John Furr, a Medicinal Chemistry Ph.D. student (1998-2000), at the University of Mississippi. Ph.D. Dissertation Title: "Mapping the NBMPR Binding Site of the *es* Nucleoside Transporter"
Current Position: Computational Chemist, Albany Molecular, Albany, NY

Amol Gupte, a Ph.D. student (8/2001-10/2006) currently in our medicinal chemistry program. Ph.D. Dissertation Title: "Probing the N^6 Position of the *es* Nucleoside Transporter Inhibitors"
Current Position: Postdoctoral Fellow, Center for Drug Discovery, University of Minnesota

Zhenxiang Zhu, Ph.D. student (7/2001-09/2006) currently in our medicinal chemistry program. Ph.D. Dissertation Title: Synthesis and Biological Evaluation of Constrained Analogs of NBMPR the Prototype *es* Nucleoside Transporter Inhibitor.
Current Position: Postdoctoral Fellow Columbia University, New York.

Wenwei Lin, Ph.D. student (9/2001-10/2006) currently in our medicinal chemistry program. Ph.D. Dissertation Title: "Design and Synthesis of Inhibitors and Probes for the *ei* Nucleoside Transporter.
Current Position: Postdoctoral Fellow, St. Jude Children's Research Hospital, Memphis, TN

Ja'Wanda Grant, Ph.D. student. Interdisciplinary Student (started 8/2002-Present). Dissertation Title: Structure-Based Design and Discovery of p53-MDM2 interaction Inhibitors as Anticancer Drugs" (Awarded NIH Minority Supplement, 2004-2006)

Surekha Pimple, Ph.D. student (8/2005-present) currently in our medicinal chemistry program. Ph.D. Working on Nucleoside transport inhibitor discovery and computer-Aided Drug Design..

Horrick Sharma, Ph.D. student (8/2006-present) currently in our medicinal chemistry program. Working on Design and Synthesis of HIV Integrase Inhibitors.

TECHNICIANS SUPERVISED

Name of Tech: J'Lynn Howell
Degree: MS.
Date Started: August, 1999
Date Ended: July, 2000

Name of Tech: Yaqin Zhang
Degree: MD.
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Date Ended: July, 2001

Name of Tech: Yuping Xiao
Degree: MS.
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Name of Tech: Bing Chen
Degree: BS.
Date Started: July, 2002
Date Ended: August, 2003

Name of Tech: Peihong Guan
Degree: MS.
Date Started: October, 2003
Date Ended: April, 2007

UNDERGRADUATE RESEARCH ASSISTANTS

DeShonda Lee a pre-pharmacy student for one year in 1995 (Work Study), completed Pharm.D. program at Xavier University of Louisiana, New Orleans.

Mauriel Clay, undergraduate chemistry major, 1995 (Work Study).

Tara Cameron, a pre-pharmacy student, 1995/96, completed Pharm.D. program at the University of Mississippi.

Wade Walker, University of Mississippi P-3 pharmacy students who worked with the applicant in the summer of 1995, is now a pharmacist.

Jennifer Boyd, University of Mississippi P-3 pharmacy student who worked with the applicant in the summer of 1996, is now a pharmacist.

Musah Ceesay, a pre-pharmacy student, summer of 1996, has completed Pharm. D. program at the University of Mississippi.

Larry Pilcher, a P-3 pharmacy student, 1998/99, has completed the Pharm. D. program at University of Mississippi.

Nia Avant (Minority, African-American), University of Mississippi P-3 Pharm.D. student, 1999/00, who was awarded an American Foundation of Pharmaceutical Education (AFPE) Undergraduate Research Scholarship under my supervision, has completed the Pharm. D. program at University of Mississippi.

Wesley Woodard, a Pharm. D. student of the University of Tennessee conducted research on NIH grant No. CA80730.

Darius Mason (Minority, African-American), a Pharm. D. student of the University of Tennessee, currently conducting research on my NIH grant No CA100102 (Awarded NIH Minority Supplement).

Rhonda Garner (Minority, African-American), a Pharm. D. student of the University of Tennessee, currently conducting research on my NIH grant No CA101856.

Marian Ores (Minority, African-American), a Pharm. D. student of the University of Tennessee, currently conducting research on my NIH grant No CA100102.

Nikita Wilson (Minority, African-American), a Pharm. D. student of the University of Tennessee, currently conducting research on my NIH grant No CA101856.

Kimberly Walker (Minority, African-American), a Pharm. D. student of the University of Tennessee, currently conducting research on my NIH grant No CA100102.

Elizabeth Ambe (Minority, African-American), a Pharm. D. student of the University of Tennessee, currently conducting research on my NIH grant No CA101856.

Professional Pharmacy (Pharm.D.) Student Advising

I served as Faculty Adviser to twelve (12), P-3 to P-4 pharmacy students from 1999-2000, while they went through the two year transition at the University of Mississippi, Oxford campus before going to the University of Mississippi Medical Center in Jackson, MS to continue the Pharm.D. Program.

Mentor for Federally Funded Ronald McNair Undergraduate Research Scholarship Program

Cornelius Varnado, a chemistry major from Alcorn University, MS (Summer 1997), University of Mississippi

Andrea Barr, a biology major from Buffalo University, NY (Summer 1998), University of Mississippi

Henry McGee, biology major from Toogaloo University, MS (Summer, 1998), University of Mississippi

Edley Destine', chemistry major from Bethel College, TN (Summer 2001), University of Tennessee

London Adams, biology major from Lane College, TN (Summer 2002), University of Tennessee

Mentor of NIH Sponsored Summer Undergraduate Research Interns

Received a five-year NIH NHLBI Short-Term Research Training for Undergraduates (T35M, 5/99-8/04) Award from NIH and served as PI and program director for two years (1999 and 2000) before leaving the University of Mississippi to move to the University of Tennessee. The specific students mentored were:
Alicia Andrews, University of Mississippi student, Summer of 1999
James Eledge, University of Southern Mississippi student, Summer of 1999
MacShelle Stewart, Jackson State University student, Summer of 2000
Kimberly Powell, Alcorn State University student, Summer of 2000
Kwanza Carter, University of Mississippi student, Summer of 2000

OTHER UNDERGRADUATES MENTORED

Deryk Cooper, Summer Student in College of Pharmacy Minority Center of Excellence Program (2004)
Verneka Murphy, Summer Student in College of Pharmacy Minority Center of Excellence Program (2005).
Jessica Nguyen, Summer Student in College of Pharmacy Minority Center of Excellence Program (2006).

Mentor in the Summer Undergraduate Research Interns at the University of Tennessee

NIH-sponsored summer biomedical research internship program directed by Dr. Edward Schneider, Department of Physiology.
Student mentored: Misty Roberts, a Sophomore from Tennessee Technology University, in the Summer of 2001.

High School Student Mentoring at the University of Mississppi

Participated in mentoring NIH-funded Biomedical Research Internship high school students while at the University of Mississippi; these were:
Thomas Taylor in the Summer of 1995
Kelvin Wellingham in the Summer of 1996
Tamberneshia Willis in the Summer of 1998

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A synthetic glycine-extended bombesin analogue interacts with the GRP/bombesin receptor

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Abstract

α-amidation of a peptide (which takes place from a glycine-extended precursor) is required to produce biologically active amidated hormones, such as gastrin-releasing peptide (GRP)/Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (bombesin). It was shown that glycine-extended gastrin mediates mitogenic effects on various cell lines by interacting with a specific receptor, different from the classical CCK_1 or CCK_2 receptors. On the basis of this observation, we have extended the concept of obtaining active glycine-extended forms of others amidated peptides to produce new active analogues. In this study, we have tested the biological behaviour of a synthetic analogue of the glycine-extended bombesin (para-hydroxy-phenyl-propionyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-OH or JMV-1458) on various in vitro models. We showed that compound JMV-1458 was able to inhibit specific (3-[^{125}I]iodotyrosyl^{15}) GRP ([^{125}I]GRP) binding in rat pancreatic acini and in Swiss 3T3 cells with K_i values of approximately 10^{-8} M. In isolated rat pancreatic acini, we found that JMV-1458 induced inositol phosphates production and amylase secretion in a dose-dependent manner. In Swiss 3T3 cells, the glycine-extended bombesin analogue dose-dependently produced [^{3}H]thymidine incorporation. By using potent GRP/bombesin receptor antagonists, we showed that this synthetic glycine-extended bombesin analogue induces its biological activities via the classical GRP/bombesin receptor. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Gastrin-releasing peptide; Bombesin, synthetic glycine-extended form; (In vitro); Biological effect; Gastrin-releasing peptide/bombesin receptor antagonist

1. Introduction

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (bombesin) is a tetradecapeptide isolated from skin frogs (Anastasi et al., 1971), whose mammalian homolog is gastrin-releasing peptide (GRP), originally isolated from porcine non-antral gastric tissue (McDonald et al., 1979). The C-terminal 14 amino acid residues of mammalian GRP are very similar to amphibian bombesin, especially the C-terminal heptapeptide (McDonald et al., 1979). Three separate receptors capable of binding bombesin have been isolated from various human cells: the GRP-preferring receptor found in the central nervous system and the gastro-intestinal tract; the neuromedin B-preferring receptor, which is also found in the central nervous system but with a more limited distribution in the gut; the bombesin receptor subtype-3 (bombesin BB_3 receptor), present in testis and lung cancer (Corjay et al., 1991; Fathi et al., 1993). Recently, a fourth member of the bombesin receptor family (bombesin BB_4 receptor) was isolated from a *Bombina orientalis* brain cDNA library (Nagalla et al., 1995). At present, the mammalian equivalent of the bombesin BB_4 receptor has not been identified (Katsuno et al., 1999). The GRP receptor is a 384-amino-acid protein

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that is glycosylated to become a 75–85 kDa cell surface receptor (Corjay et al., 1991). Activation of this receptor results in stimulation of phospholipase C with subsequent increases of inositol phosphates and intracellular Ca^{2+} (Corjay et al., 1991; Kroog et al., 1995). Mammalian bombesin-like peptides elicit a broad spectrum of biological responses, including secretion of gastrointestinal, adrenal and pituitary hormones, gastric acid and mucous secretion, regulation of smooth muscle contraction, and modulation of neuronal firing rate. In the central nervous system, these peptides are thought to influence regulation of homeostasis, thermoregulation, metabolism and behaviour (Tache et al., 1988; Lebacq-Verheyden et al., 1990). Bombesin family peptides can also act as mitogens. This has been shown in Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983) and in human small cell lung carcinoma where the peptides have been implicated in a subset of tumors to function in autocrine growth loops (Cuttitta et al., 1985).

Prepro GRP is converted to bioactive amidated GRP by sequential enzymatic steps: trypsin-like/subtilisin-like cleavage, carboxypeptidase-like processing and formation of the glycine-extended intermediate, which serves as a substrate for peptidyl glycine α-amidating monooxygenase (Cuttitta, 1993).

Like other amidated peptides, gastrin is synthetized as a precursor that undergoes posttranslational processing to an amidated product on the C-terminus. Posttranslational processing intermediates of gastrin, specifically glycine-extended gastrin (gastrin-Gly), serve as substrate for the amidation reaction (Dockray et al., 1996). Whereas for many years, amidation of gastrin was thought to be an essential prerequisite for biological activity, recent results indicate that non-amidated gastrins can also stimulate cells proliferation in vitro (Seva et al., 1994; Singh et al., 1995). Interestingly, it was shown that proliferative effects induced by the glycine-extended progastrin were mediated through a specific receptor, different from the classical CCK_1 or CCK_2 receptors.

On the basis of the data obtained with the glycine-extended progastrin, we hypothesized that the concept of obtaining active glycine-extended forms of amidated peptides could be generalized to other amidated peptide hormones. As an example, we decided to investigate the biological activities of Gly-extended forms of bombesin. In this study, we present the biological behaviour of a synthetic analogue of the glycine-extended bombesin (para-hydroxy-phenyl-propionyl-Gln-Trp-Ala-Val-Gly-His-Leu-Met-Gly-OH or JMV-1458) on various in vitro models. We have compared JMV-1458 and amidated bombesin for their ability to induce inositol phosphates production and amylase release in rat pancreatic acini and to stimulate DNA synthesis in Swiss 3T3 cells. By using specific GRP/bombesin receptor antagonists, we have studied the pharmacological profile of the JMV-1458-activated receptor.

2. Materials and methods

2.1. Chemicals

Collagenase EC 3.4.24.3 was obtained from Serva (Heidelberg, FRG). NaCl, KCl, NaH_2PO_4, $MgCl_2$, $MgSO_4$, $CaCl_2$, K_2CO_3, LiCl, KH_2PO_4, $NaHCO_3$, sodium pyruvate, sodium glutamate, glutamine, ammonium formate, glucose, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), trypsin inhibitor, trichloroacetic acid and Krebs–Henseleit buffer were from Sigma (St. Louis, MO, USA). Bovine serum albumin fraction V was from Euromedex (France). The protein concentration was evaluated using the Bio-Rad protein assay (Bio-Rad, Richmond, CA, USA), based on the Bradford dye-binding procedure. Dowex AG1-X8 anion exchange resin (100–200 mesh, formate form) was also from Bio-Rad. Phadebas amylase test reagent was from Pharmacia (France). (3-[^{125}I]iodotyrosyl^{15}) GRP ([^{125}I]GRP; 2000 Ci/mmol), *myo*-[2-^{3}H]inositol (16.5 Ci/mmol) and [^{3}H]thymidine (24 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Glutamine, penicillin/streptomycin, fetal bovine serum, amino acid mixture and essential vitamin mixture were from Gibco Life Technologies (Scotland). Dulbecco's modified Eagles' medium (DMEM) was from Bio Whittaker (Verviers, Belgium).

Bombesin, JMV-1458, D-Phe-Gln-Trp-Ala-Val-Gly-His-*N*-(1-amino-1-isobutyl-2-hydroxy-hexane) (JMV-641), *N*-((para-hydroxy-phenyl)-propionic)-Gln-Trp-Ala-*N*-3 (*S*) amino-2-oxo-1-azepine acetic)-His-Leu-methyl ester (JMV-1799) and *N*-((para-hydroxy-phenyl)-propionic)-Gln-Trp-Ala-*N*-(3 (*S*) amino-2-oxo-1-azepine acetic)-His-(1-amino-1-isobutyl-2-hydroxy-hexane) (JMV-1802; Fig. 1) were synthesized in our laboratory. A stock solution of each bombesin analogue was prepared in pure dimethyl sulfoxide and stored at −20°C. Dilutions were made in experiment incubation medium and the maximal final concentration did not contain more than 1% dimethyl sulfoxide.

2.2. Experiments on dispersed rat pancreatic acini

2.2.1. Preparation of dispersed rat pancreatic acini

Male Wistar rats (200–300 g) were obtained from the Pharmacological Breeding Center of Montpellier University (France). Dispersed acini were prepared as previously described (Peikin et al., 1978) with some modifications (Jensen et al., 1982) in buffer 1 containing (in mM) HEPES (pH 7.4) 25.5, NaCl 98, KCl 6, NaH_2PO_4 2.5, sodium pyruvate 5, sodium glutamate 5, glutamine 2, $CaCl_2$ 1.5, glucose 11.5, $MgCl_2$ 1, trypsin inhibitor 0.01% (p v^{-1}), amino acid mixture 1% (v v^{-1}) and essential vitamin mixture 1% (v v^{-1}).

Bombesin

Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

JMV-1458

JMV-641

H-D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu-ψ (CHOH)-$(CH_2)_3$-CH_3

JMV-1799

JMV-1802

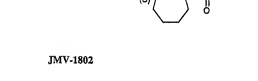

Fig. 1. Structures of the bombesin analogues used in the various experiments.

2.2.2. Binding of [^{125}I]GRP

The incubation medium used for binding experiments (buffer 2) was Krebs–Henseleit buffer (pH 7.4) supplemented with 1% bovine serum albumin (p v^{-1}). For displacement experiments, dispersed rat acini (0.5 mg protein) were incubated with 20 pM of [^{125}I]GRP (1/10 K_d) for 60 min at 37°C in a final volume of 0.5 ml in the presence of various concentrations of bombesin analogues in polypropylene tubes. Non-specific binding was determined in the presence of 10 μM unlabeled bombesin and was always less than 25% of the total binding. Incubation was terminated by adding 3 ml of buffer 2 at 4°C supplemented with 4% bovine serum albumin (p v^{-1}). Aliquots were then centrifuged at 4°C for 10 min at 3000 rpm. The supernatants were discarded and the radioactivity bound to the pellet was measured. Incubations were performed in duplicate and mean values were used for calculations.

2.2.3. Measurement of inositol phosphates production

Intracellular inositol phosphates were determined according to Qian et al. (1993) with some modifications. Rat pancreatic acini (6 mg protein ml^{-1}) were incubated in buffer 1 with 400 μCi *myo*-[2-^{3}H]inositol for 2 h at 37°C. Acini were then washed three times in the same buffer and incubated (15 min, 37°C) in 20 ml of buffer 3 (buffer 3 contained (in mM) HEPES (pH 7.4) 20, NaCl 116, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 1, glucose 11, $MgSO_4$ 1.2, $NaHCO_3$ 5) supplemented with 20 mM LiCl. Aliquots of [^{3}H]inositol-loaded acini (0.4 ml) were incubated (15 min, 37°C) with buffer 3 or with various compounds as described in each individual experiment in a final volume of 0.5 ml. The incubation was terminated by adding 500 μl $HClO_4$ 5% (v v^{-1}). Each tube subsequently received 155 μl of K_2CO_3 2 M and 25 μl of HEPES 0.4 M, and the contents were vortexed and centrifuged (3000 rpm, 10 min). 900 μl of each tube were collected and applied to a column containing 1.6 ml of a 1:4 (p p^{-1}) Dowex AG-1-X8 anion-exchange resin in distilled water. The columns were washed in the following manner: 10 ml distilled water, 4 ml of 40 mM ammonium formate. Inositol phosphates were eluted with 5 ml of 1 M ammonium formate. The eluates were then assayed for their radioactivity after the addition of 10 ml Complete Phase Combining System for liquid scintillation counting solution to each vial.

2.2.4. Amylase release test

Dispersed acini (0.5 mg) were suspended in 0.5 ml of buffer 1 and incubated for 30 min at 37°C in the same buffer. Amylase release was measured as previously described (Jensen et al., 1978; Sekar et al., 1991) using the Phadebas reagent (Ceska et al., 1969). Incubations were performed in duplicate and mean values were used for calculations.

2.3. Experiments on Swiss 3T3 cells

2.3.1. Cells culture

Swiss 3T3 cells were a gift from Dr. Abello (Inserm U45, Lyon, France). Cells were maintained at 37°C in a humidified atmosphere containing 10% CO_2 by serial passages in DMEM supplemented with 10% fetal bovine serum, 1 nM glutamine and 1% (v v^{-1}) penicillin/streptomycin.

2.3.2. Binding of [^{125}I]GRP

The day before the binding experiment, cells were seeded into 24-well plates (10^5 cells per well) and incubated for 24 h at 37°C in maintenance medium. After 24 h incubation time, cells were incubated with 20 pM of [^{125}I]GRP (1/10 K_d) for 60 min at 22°C in 1 ml of maintenance medium without fetal bovine serum supplemented with 0.2% bovine serum albumin (p v^{-1}), in the presence of various concentrations of bombesin analogues. Non-specific binding was determined in the presence of 10 μM unlabeled bombesin and was always less than 15% of the total binding. After 1 h incubation, cells were

washed twice with cold phosphate buffer saline supplemented with 0.2% bovine serum albumin (p v^{-1}) and solubilized in 1 ml of 1 M NaOH. Samples were removed from the plates, placed in tubes and associated radioactivity was determined. Incubations were performed in duplicate and mean values were used for calculations.

2.3.3. Swiss 3T3 cells proliferation studies by [^{3}H]thymidine incorporation

Swiss 3T3 cells (10^5 cells) were plated in 1 ml of maintenance medium DMEM supplemented with 10% fetal bovine serum (v v^{-1}) and allowed to attach overnight. Then, cells were cultured for 24 h in serum free medium supplemented with 0.2% bovine serum albumin (p v^{-1}). Cells were then treated for 24 h with various concentrations of test compounds. DNA synthesis was estimated by measurement of [^{3}H]thymidine incorporation into trichloroacetic acid precipitable material. The [^{3}H]thymidine (0.5 mCi per well) was added during the last hour of the 24 h treatment period for 4 h duration. Then, the cells were washed twice with phosphate buffer saline supplemented with 0.2% bovine serum albumin (p v^{-1}) to remove unincorporated [^{3}H]thymidine. DNA was precipitated with 5% trichloracetic acid (p v^{-1}) at 4°C for 30 min. Precipitates were washed twice with 95% ethanol, dissolved in 1 ml of 1 M NaOH and analyzed in a liquid scintillation counter after neutralization with 1 ml of 1 M HCl. Incubations were performed in duplicate and mean values were used for calculations.

3. Results

3.1. [^{125}I]GRP binding experiments

The effects of some bombesin analogues on [^{125}I]GRP binding were investigated. Bombesin, JMV-1458, JMV-641 (Azay et al., 1996; Llinares et al., 1999), JMV-1799, and JMV-1802 were tested for their potency to inhibit specific binding of [^{125}I]GRP to rat pancreatic acini and Swiss 3T3 cells. In rat pancreatic acini, the amidated bombesin exhibited high affinity for bombesin receptor ($K_i = 1.8 \pm 0.8$ nM); the synthetic glycine-extended bombesin analogue JMV-1458 had a 10-fold lower affinity ($K_i = 15 \pm 3$ nM) (mean ± S.D. from three independent experiments performed in duplicate; Fig. 2A). However, the glycine-extended bombesin was only moderately active ($K_i = 106 \pm 10$ nM). In Swiss 3T3 cells, bombesin and JMV-1458 inhibited [^{125}I]GRP binding in a dose-dependent manner with K_i values, respectively, of 1.6 ± 0.7 and 21 ± 12 nM (mean ± S.D. from three independent experiments performed in duplicate; Fig. 2B). Again, the glycine-extended bombesin was not very potent in inhibiting [^{125}I]GRP binding to Swiss 3T3 cells ($K_i = 500 \pm 21$ nM). As shown

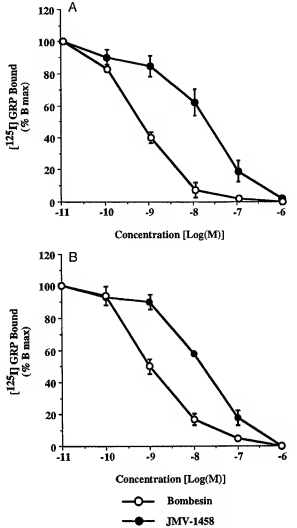

Fig. 2. Binding experiments of [^{125}I]GRP on rat pancreatic acini (A) and Swiss 3T3 cells (B). The specific binding of [^{125}I]GRP was measured in the presence of various concentrations of bombesin (○) and JMV-1458 (●). Results are the means ± S.D. of three experiments, each performed in duplicate.

in Table 1, the three potent bombesin receptor antagonists JMV-641, JMV-1799 and JMV-1802 were able to inhibit [^{125}I]GRP binding in a dose-dependent manner both on rat pancreatic acini and Swiss 3T3 cells with high affinities (K_i values of JMV-641, JMV-1799 and JMV-1802 were, respectively, 1.0 ± 0.1, 3.1 ± 2.1 and 3.8 ± 2.9 nM in rat pancreatic acini and 0.8 ± 0.1, 1.2 ± 0.6 and 0.8 ± 0.4 nM in Swiss 3T3 cells; mean ± S.D. from three independent experiments performed in duplicate).

3.2. Measurement of inositol phosphates production in dispersed rat pancreatic acini

It is well known that following bombesin binding, a series of early events occurs, including inositol phosphates

Table 1
K_i values of various bombesin analogues to inhibit [^{125}I]GRP binding in rat pancreatic acini and Swiss 3T3 cells. Results are the means ± S.D. of three independent experiments, each performed in duplicate

Compounds	K_i (nM)	
	Rat pancreatic acini	Swiss 3T3 cells
JMV-641	1.0 ± 0.1	0.8 ± 0.1
JMV-1799	3.1 ± 2.1	1.2 ± 0.6
JMV-1802	3.8 ± 2.9	0.8 ± 0.4

Table 2
K_i values of various bombesin receptor antagonists on inositol phosphates production induced by 10^{-7} M bombesin and 10^{-6} M JMV-1458 in rat pancreatic acini. Results are the mean ± S.D. of three independent experiments, each performed in duplicate

Compounds	K_i (nM)	
	Bombesin-induced inositol phosphates production	JMV-1458-induced inositol phosphates production
JMV-641	0.19 ± 0.16	0.08 ± 0.03
JMV-1799	2.40 ± 2.10	2.20 ± 2.10
JMV-1802	0.30 ± 0.17	0.38 ± 0.23

generation (Corjay et al., 1991; Kroog et al., 1995). We have tested the capacity of bombesin and of the synthetic glycine-extended bombesin analogue JMV-1458 to stimulate inositol phosphates production. We showed that these two compounds were able to stimulate inositol phosphates production in a dose-dependent manner (Fig. 3). The maximal stimulation was obtained with 10^{-7} M bombesin and with 10^{-6} M JMV-1458, both compounds having the same efficacy inducing the same maximal response. As compared to the basal value, 10^{-7} M bombesin and 10^{-6} M JMV-1458 produced, respectively, a 67.5 ± 2.5% and a 64.0 ± 2.9% increase of inositol phosphates production (mean ± S.D. from four independent experiments performed in duplicate). The effective concentrations producing 50% of the maximal response (EC_{50}) were, respectively, 2.3 ± 0.6 and 46 ± 38 nM for bombesin and JMV-1458 (mean ± S.D. from three independent experiments performed in duplicate).

We have tested the potency of the bombesin analogues JMV-641, JMV-1799 and JMV-1802 for their ability to stimulate the inositol phosphates production and to inhibit the inositol phosphates production induced by 10^{-7} M bombesin and by 10^{-6} M glycine-extended bombesin analogue JMV-1458. Our results indicated that none of the tested compounds affected the basal inositol phosphates production even at doses as high as 10^{-5} M. On the other hand, JMV-641, JMV-1799 and JMV-1802 were able to inhibit inositol phosphates production induced by bombesin and by the glycine-extended bombesin analogue in a dose-dependent manner, displaying the same inhibition profiles. The K_i values of these antagonists are reported in Table 2.

3.3. Amylase release test in dispersed rat pancreatic acini

As already described (Llinares et al., 1999), our results showed that bombesin was dose-dependently able to stimulate enzyme secretion from isolated rat pancreatic acini. In this study, we showed that the synthetic glycine-extended bombesin analogue JMV-1458 stimulated amylase release in a dose-dependent manner (Fig. 4). The maximal stimu-

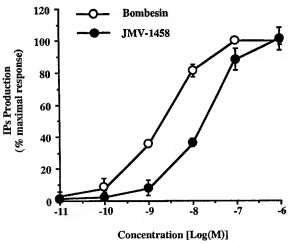

Fig. 3. Dose–response curves for bombesin (○) and JMV-1458 (●) on inositol phosphates production in dispersed rat pancreatic acini. After subtraction of the basal inositol phosphates production, data were expressed as percentage of the response obtained with 10^{-7} M bombesin or 10^{-6} M JMV-1458. For each agonist, the mean control value was 1400 dis/min with a magnitude of stimulation of 3. Results are the means ± S.D. of three experiments, each performed in duplicate.

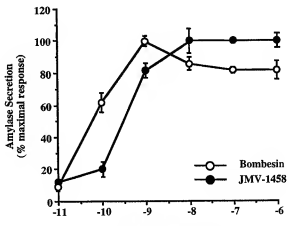

Fig. 4. Dose–response curves for bombesin (○) and JMV-1458 (●) on amylase secretion in dispersed rat pancreatic acini. After subtraction of the basal amylase secretion, data were expressed as percentage of the response obtained with 10^{-9} M bombesin and 10^{-8} M JMV-1458. For each agonist, the magnitude of stimulation was 20. Results are the means ± S.D. of three experiments, each performed in duplicate.

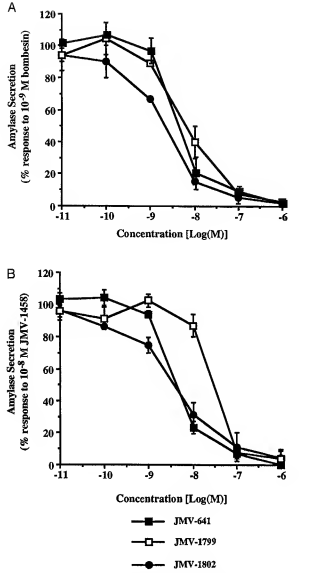

Fig. 5. Effect of JMV-641 (■), JMV-1799 (□) and JMV-1802 (●) on amylase secretion induced by 10^{-9} M bombesin (A) and 10^{-8} M JMV-1458 (B) in dispersed rat pancreatic acini. After subtraction of the basal amylase secretion, data were expressed as percentage of the response obtained with 10^{-9} M bombesin (A) and 10^{-8} M JMV-1458 (B). For each agonist, the magnitude of stimulation was 20. Results are the means ± S.D. of three experiments, each performed in duplicate.

lation was obtained with 10^{-9} M bombesin and with 10^{-8} M JMV-1458. Considering the amylase release obtained with 10^{-9} M bombesin as the reference, the JMV-1458 compound is a full agonist inducing the same maximal response. The apparent effective concentration producing 50% of the maximal amylase secretion (EC_{50}) was 0.07 ± 0.04 and 0.5 ± 0.7 nM for bombesin and JMV-1458, respectively (mean ± S.D. from three independent experiments performed in duplicate).

Compounds JMV-641, JMV-1799 and JMV-1802 were tested for their capacity to stimulate amylase release and to inhibit the amylase release induced by 10^{-9} M bombesin and 10^{-8} M JMV-1458. Our results showed that none of these compounds affected the basal amylase release even when tested at doses as high as 10^{-5} M. On the other hand, JMV-641, JMV-1799 and JMV-1802 inhibited, in a dose-dependent manner, amylase release induced by bombesin and by the glycine-extended bombesin analogue. The K_i values of JMV-641, JMV-1799 and JMV-1802 in inhibiting amylase secretion induced by bombesin were 2.1 ± 1.1, 21 ± 3 and 3.3 ± 1.1 nM, respectively (mean ± S.D. from three independent experiments performed in duplicate; Fig. 5A). The K_i values of JMV-641, JMV-1799 and JMV-1802 in inhibiting amylase secretion induced by JMV-1458 were 3.1 ± 2.2, 12 ± 5 and 2.7 ± 0.3 nM, respectively (mean ± S.D. from three independent experiments performed in duplicate; Fig. 5B).

3.4. Swiss 3T3 proliferation studies

We have tested the capacity of bombesin and the synthetic glycine-extended bombesin analogue JMV-1458 to stimulate proliferation of Swiss 3T3 cells by measuring [^{3}H]thymidine incorporation (Fig. 6). Our results showed that bombesin induced [^{3}H]thymidine incorporation in a dose-dependent manner with an EC_{50} value of 0.36 ± 0.06 nM (mean ± S.D. from three independent experiments performed in duplicate). Moreover, the glycine-extended bombesin analogue JMV-1458 was able to induce [^{3}H]thymidine incorporation in a dose-dependent manner with an EC_{50} value of 3.0 ± 1.7 nM (mean ± S.D. from four independent experiments performed in duplicate). JMV-1458 was a full agonist inducing the same maximal response than bombesin.

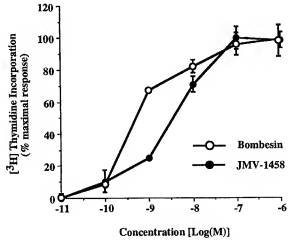

Fig. 6. Dose-response curves for bombesin (○) and JMV-1458 (●) on [^{3}H]thymidine incorporation in Swiss 3T3 cells. After subtraction of the basal [^{3}H]thymidine incorporation, data were expressed as percentage of the response obtained with 10^{-7} M bombesin and 10^{-6} M JMV-1458. For each agonist, the mean control value was 6800 dis/min with a magnitude of stimulation of 8.5. Results are the means ± S.D. of four experiments, each performed in duplicate.

Table 3
K_i values of various bombesin receptor antagonists on [^{3}H]thymidine incorporation induced by 10^{-7} M bombesin and 10^{-6} M JMV-1458 in Swiss 3T3 cells. Results are the means ± S.D. of three independent experiments, each performed in duplicate

Compounds	K_i (nM)	
	Bombesin-induced [^{3}H]thymidine incorporation	JMV-1458-induced [^{3}H]thymidine incorporation
JMV-641	0.10 ± 0.05	0.04 ± 0.01
JMV-1799	7.87 ± 1.57	4.50 ± 2.70
JMV-1802	0.47 ± 0.11	0.10 ± 0.06

We have tested compounds JMV-641, JMV-1799 and JMV-1802 for their capacity to stimulate [^{3}H]thymidine incorporation and to inhibit [^{3}H]thymidine incorporation induced by 10^{-7} M bombesin and 10^{-6} M JMV-1458 (Table 3). None of these compounds were able to stimulate [^{3}H]thymidine incorporation in Swiss 3T3 cells. However, they inhibited the [^{3}H]thymidine incorporation induced by bombesin in a dose-dependent manner with K_i values, respectively, of 0.10 ± 0.05, 7.87 ± 1.57 and 0.47 ± 0.11 nM (mean ± S.D. from three independent experiments performed in duplicate). We also demonstrated that these compounds dose-dependently inhibited JMV-1458-induced [^{3}H]thymidine incorporation with K_i values, respectively, of 0.04 ± 0.01, 4.50 ± 2.70 and 0.10 ± 0.06 nM (mean ± S.D. from three independent experiments performed in duplicate).

4. Discussion

On the basis of the results obtained by some authors on glycine-extended forms of gastrin (Seva et al., 1994; Singh et al., 1995), we have tried to generalize this concept of obtaining active glycine-extended forms of other amidated peptides. In this study, we presented the behaviour of a synthetic glycine-extended bombesin analogue (compound JMV-1458) on various biological effects.

Binding experiments indicated that compound JMV-1458 inhibited specific [^{125}I]GRP binding in rat pancreatic acini and in Swiss 3T3 cells with K_i values of approximately 10^{-8} M. These results indicate that JMV-1458 interacts with the classical GRP/bombesin receptor in rat pancreatic acini and in Swiss 3T3 cells. These binding results differs from those obtained by Seva et al. (1994) or Singh et al. (1995) in the sense that they showed that glycine-extended gastrin (2–17) occupies a different binding site than gastrin (2–17). We have already shown that JMV-1458 is specific for the GRP/bombesin receptor subtype. This compound presents a weak affinity (in the micromolar range) for both neuromedin-B and bombesin BB_3 receptor subtypes (data not shown). However, the glycine-extended form of bombesin was only moderately active at the GRP/bombesin receptor.

To study JMV-1458 behaviour in more details, we have tested its ability to induce inositol phosphates production and amylase release in rat pancreatic acini and to stimulate DNA synthesis in Swiss 3T3 cells.

In rat pancreatic acini, we have demonstrated that bombesin and the synthetic glycine-extended bombesin analogue JMV-1458 dose-dependently induced inositol phosphates production and amylase secretion. Compound JMV-1458 was about 10 times less potent that bombesin. Like bombesin, we showed that glycine-extended bombesin analogue JMV-1458 stimulated [^{3}H]thymidine incorporation in a dose-dependent manner in Swiss 3T3 cells. We found an EC_{50} value of approximately 10^{-9} M for the glycine-extended bombesin analogue. In the same cellular model, Mervic et al. (1991) showed that the concentration of bombesin-Gly required to half maximally stimulate [^{3}H]thymidine uptake was 1300 nM. In view of these results, we can hypothesize that the N-terminal modification included in compound JMV-1458 conferred to this glycine-extended bombesin analogue a better affinity for the GRP/bombesin receptor.

Carboxyamidation is a key event in the biosynthetic maturation of peptides. The C-terminal amide function has been shown in a large variety of amidated peptide hormones to be crucial for the expression of biological activity (Hilsted and Rehfeld, 1986). This is particularly true for gastrin (Martinez et al., 1986) and GRP/bombesin (Heimbrook et al., 1989), where suppression of the C-terminal amide resulted in potent antagonist compounds. We have shown in this study that a synthetic glycine-extended bombesin analogue was able to induce, with high potency, the same biological effects than bombesin, its amidated counterpart, on various models. However, whereas glycine-extended forms of gastrin seem to interact with binding sites different from that of gastrin (Seva et al., 1994; Singh et al., 1995), in our case, the glycine-extended analogue of bombesin seems to interact with the same binding sites than the natural amidated peptide. To assess this assumption, we have studied the inhibition profiles of JMV-641, JMV-1799 and JMV-1802 (three potent bombesin receptor antagonists) on inositol phosphates production and amylase release from rat pancreatic acini and on [^{3}H]thymidine incorporation in Swiss 3T3 cells induced by bombesin and JMV-1458. We first tested the capacities of JMV-641 (Azay et al., 1996; Llinares et al., 1999), JMV-1799 and JMV-1802 to inhibit [^{125}I]GRP binding on rat pancreatic acini and on Swiss 3T3 cells. We showed that JMV-641, JMV-1799 and JMV-1802 interacted with the GRP/bombesin receptor with high affinities. These compounds were then tested for their ability to inhibit bombesin-induced inositol phosphates production and amylase release in rat pancreatic acini and for their capacity to inhibit bombesin-induced [^{3}H]thymidine incorporation in Swiss 3T3 cells. On these various models, we

showed that JMV-641, JMV-1799 and JMV-1802 were very potent GRP/bombesin receptor antagonists. We decided to use these compounds to test the implication of the GRP/bombesin receptor on JMV-1458-induced inositol phosphates production and amylase secretion in rat pancreatic acini and on JMV-1458-induced [^{3}H]thymidine incorporation in Swiss 3T3 cells. Our results showed that compounds JMV-641, JMV-1799 and JMV-1802 were very potent in inhibiting the biological effects induced by the glycine-extended analogue of bombesin.

Whatever the agonist tested (bombesin or the glycine-extended analogue of bombesin JMV-1458), we found that the bombesin receptor antagonists JMV-641, JMV-1799 and JMV-1802 inhibited, in a dose-dependent manner, inositol phosphates production and amylase secretion in rat pancreatic acini, as well as [^{3}H]thymidine uptake in Swiss 3T3 cells. In each experiment, these antagonists displayed the same inhibition profile showing similar K_i values. In view of these results, we concluded that the synthetic glycine-extended bombesin analogue JMV-1458 exerted its biological activities by interacting with the classical GRP/bombesin receptor.

In summary, although it was described that carboxyamidation was essential for biological activity of peptide hormones, our results showed that a synthetic analogue of the glycine-extended bombesin was able to induce inositol phosphates production and amylase secretion in rat pancreatic acini and [^{3}H]thymidine accumulation in Swiss 3T3 cells with high potency. We also concluded that an amidated C-terminal residue is not essential for obtaining high affinity and potency in bombesin. Moreover, we showed that biological effects induced by this glycine-extended bombesin analogue were antagonized by very potent GRP/bombesin antagonists, suggesting that compound JMV-1458 interacts with the classical GRP/bombesin receptor.

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A COMBINATORIAL PEPTOID LIBRARY FOR THE IDENTIFICATION OF NOVEL MSH AND GRP / BOMBESIN RECEPTOR LIGANDS

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ABSTRACT

A tripeptoid library was synthesized using 69 different primary amines in initially 69 individual reactions by the mix and split approach. The resulting library consisted of 328,509 (69^3) single compounds, divided in 69 subpools each containing 4,761 entities. The 69 subpools were tested in two binding assays, one for α-MSH (α-melanotropin) and one for GRP (gastrin-releasing peptide)/bombesin. The sublibraries with the highest affinity to the MSH receptor (i.e. melanocortin type 1 or MC1 receptor) and, respectively, the GRP-preferring bombesin receptor were identified by an iterative process. Individual tripeptoids with good binding activity were resynthesized, analyzed and their dissociation constants and biological activity determined. The K_D of the most potent MC1 receptor ligand was 1.58 µmol/l and that of the GRP-preferring bombesin receptor 3.40 µmol/l. Extension of this latter tripeptoid by one residue at the N-terminus led to the identification of a tetrapeptoid structure whose K_D value increased to 280 nmol/l. A similar increase in activity was not observed with the most potent MSH tripeptoid ligand when extended by one residue, but a compound suitable for radioiodination and lacking the N-terminal amino group had a slightly higher binding activity than the tripeptoids (K_D ~ 850 nmol/l). These results demonstrate that testing a peptoid library containing 328,509 single compounds led to the successful identification of new ligands for both the MC1 receptor as well as the GRP-preferring bombesin receptor.

INTRODUCTION

Combinatorial compound libraries (CCL) of natural and non-natural entities provide a useful tool for the identification of novel agonists and antagonists. The application of combinatorial methods has led to the identification of a variety of ligands for cellular receptors [1, 2]. Since peptide oligomers in general show low half-life times in the circulation due to their susceptibility to proteases, the concept of molecular diversity was extended to non-natural backbones. Oligo-N-substituted glycines, also called peptoids, provide a class of non-natural compounds which can be synthesized in an effective manner and show good stability to proteases [3], even if present only as peptoid element within a peptide structure [4]. Therefore, peptoid libraries were amongst the first CCLs used for screening [5, 6], leading to the identification of potent ligands, e.g. for the α_1-adrenergic and the μ-opiate receptor [7, 8]. Many more recent applications [9, 10] followed these earlier studies because of the great variation of structures that can be generated in peptoid libraries, based on the numerous primary amines which are commercially available [6]. This makes it possible to limit the molecular size of the compounds to a few residues whilst achieving the same level of diversity as with somewhat longer peptide chains. The advantage of shorter oligomers is that low-molecular compounds generally have better tissue penetration than larger-size molecules.

α-Melanotropin (α-MSH) is well known to stimulate melanocytes and melanoma cells which express type 1 melanocortin (MC1) receptors [11, 12]. MSH ligands are extensively studied because of their potential use for melanoma localization and treatment [12]. In order to avoid any tumorigenic action during application of such compounds, MSH derivatives with antagonist activity would be particularly useful for this purpose. Likewise, GRP-preferring bombesin receptors, which are expressend on pancreatic tumor and small cell lung carcinoma cells and other types of tumors [13], are potential targets for tumor diagnosis and therapy. Although peptide antagonists for both the MSH receptor [14] and the GRP-preferring bombesin

receptor [15] have been described, it is well recognized that peptide ligands of this size usually suffer from relatively short half-lifes in the circulation. Also, MSH and bombesin generally bind to several receptor subtypes. As an alternative, low-molecular weight ligands which are stable in the circulation and exhibit good tumor penetration are currently being sought for application in tumor research.

The purpose of this study was to employ the molecular diversity approach in order to identify novel peptoid ligands from a CCL. In order to produce low-molecular weight ligands, we restricted the length of the oligomeric structure to three or four residues. The different compounds were tested with binding assays for MSH using human melanoma cells and, respectively, for GRP/bombesin using rat pancreatic carcinoma cells.

MATERIALS AND METHODS

Materials

Solvents were obtained from Fluka (Buchs, Switzerland) and Merck (Darmstadt, Germany) and were used without further purification. Reagents for the syntheses were purchased from Fluka and Aldrich (Buchs, Switzerland), and EMKA-Chemie (Markgröningen, Germany). Fmoc Rink polystyrene resin [16] for library synthesis and the resynthesis of individual peptoids was obtained from Novabiochem (Läufelfingen, Switzerland). [Nle^4, D-Phe^7]-α-MSH and [Tyr^4]-bombesin were purchased from Bachem (Bubendorf, Switzerland). Soybean trypsin inhibitor (SBTI), phenylmethanesulfonly fluoride (PMSF) and leupeptin were obtained from Fluka (Buchs, Switzerland). Cell culture reagents originated from Gibco Life Technologies (Basel, Switzerland).

Analytics

Analytical and preparative HPLC was performed on a Jasco HPLC instrument (Tokyo, Japan) equipped with autoinjector, two pumps and a

UV monitor. Analytical HPLC was carried out on a Waters Symmetry C_{18} column (3,9 x 150 mm) using a flow rate of 1 ml/min and a linear solvent gradient from 5% to 100% solvent B in 20 minutes (solvent A = 0.1% TFA in water; solvent B = 0.1% TFA in acetonitrile/H_2O 7:3). Preparative HPLC was performed on a Waters Symmetry C_{18} column (19 x 150 mm) with a flow rate of 5 ml/min. Mass spectrometry analysis was done on a Linear Scientific 1700 MALDI system (Reno, NV, U.S.A.) at Ciba-Geigy (Basel, Switzerland).

Instrument for Syntheses

The assembly of the peptoid library and the resyntheses of individual peptoids were performed on a semi-automated synthesizer (designed by Ciba-Geigy, Basel, Switzerland), equipped with a glass vessel, a glass filter frit at the bottom and teflon tubes at both ends of the vessel. The solvents entered the reaction chamber through the top of the vessel, following evacuation of the chamber and subsequent opening of a valve separating the storage vessel of the appropriate solvent from the reaction chamber. Coupling mixtures and wash solvents were removed from the resin by filtration through the glass filter at the bottom of the reaction vessel into a waste vessel.

General Synthesis Procedures

Cleavage of Fmoc groups: The Fmoc protecting groups were removed from the resin by treatment with 20% piperidine/80% DMF (2 x 5 min; 1 x 20 min). Remaining traces of piperidine were removed by washing the resin 3x with DMF, 1x with isopropyl alcohol and again 2x with DMF.

Bromoacetylation: Free amino groups on the resin were treated with 10 equivalents of bromoacetic acid (0.6 M in DMF) and 12.5 equivalents of diisopropylcarbodiimide (3.2 M in DMF) for 30 minutes. The resin was washed with DMF and the reaction was repeated. After bromoacetylation the resin was washed several times with DMF and 2x with DMSO.

Nucleophilic displacement reaction with amines: Bromoacetylated resins were agitated with 40 equivalents of the different amines, added as 1-2 M solutions in DMSO (the final concentration was dependent on the solubility of the corresponding amine). The resins were treated with the amine solutions for 2 hours, washed with DMSO, DMF, isopropyl alcohol and again twice with DMF.

Distribution of the resin in equal portions: The resin was portioned according to the isopycnic slurry method. To this end, the resin was suspended in dichloromethane/DMF 4:1. Equal volume portions of the resin suspension were distributed to the different reaction vessels. In order to keep the distribution errors small, the resin was distributed in two steps. First 90% of the resin were distributed, then the remaining 10% were diluted and distributed to the reaction vessels.

Cleavage of the peptoids from the resin: The peptoid-bearing resins were treated with 90% TFA/10% H_2O (1 x 10 min, 1 x 20 min; 100 ml cleavage solution for 10 mmol peptoid loading on the resin). After cleavage, the resin was washed with a small amount of trifluoroethanol.

Synthesis of the Original Library

The Fmoc group of 4.8 mmol Fmoc Rink amide polystyrene resin (loading 0.48 mmol/g) was removed and the free amino groups were bromoacetylated (see above). Then the resin was distributed in 69 equal portions to polyethylene syringes equipped with a porous ethylene disk, followed by the nucleophilic displacement reaction (see above). All resin portions were combined and the bromoacetylation, distribution in equal portions and the nucleophilic displacement reaction were repeated twice to yield 69 trimeric peptoid mixtures of the general structure A-X-X, with A as a defined peptoid unit and X as a mixture of all possible N-alkylated glycine moieties. All 69 peptoid mixtures, each containing 4,761 individual compounds, were individually cleaved from the resin and the solutions

placed in Eppendorf tubes. The cleavage solutions were evaporated by storage in an evacuated desiccator over potassium hydroxide for at least 12 hours. The residues were then lyophilyzed twice from 90% AcOH/10% H_2O, dissolved in DMSO to give 0.05 M solutions (total peptoid content) and stored at 4°C.

Synthesis of the Deconvolution Libraries

A first generation deconvolution library was generated by removing the Fmoc protecting group of 0.36 mmol Fmoc Rink amide polystyrene resin (loading 0.48 mmol/g). The resin was bromoacetylated, distributed into 69 equal portions and the bromine displaced by the different amines in a nucleophilic reaction (details see above). After combining all resins, this reaction sequence was repeated. For the introduction of the third N-alkylated glycine unit, which was the one which gave the best result in the biological testing of the original library, all 69 dipeptoid mixtures were individually bromoacetylated and then the bromine displaced with a single amine to produce trimeric structures of the general structure A-B-X with A and B as defined building blocks and X as a random mixture of all possible N-alkylated glycines. Again the mixtures were individually cleaved from the resin, the solvents removed in a desiccator and the residues lyophilized twice from 90% AcOH/10% H_2O. These residues were then dissolved in DMSO to yield 0.05 M solutions of the peptoid mixtures, ready for the biological testing.

For the second deconvolution step, the Fmoc protecting group of 0.36 mmol Fmoc Rink amide polystyrene resin (loading 0.48 mmol/g) was removed and the resin bromoacetylated. After distribution into 69 equal portions and the nucleophilic displacement of the bromine by the amines, each resin portion was treated with two defined N-alkylated units by two cycles of bromoacetylation and nucleophilic displacement reaction. After cleavage from the resin, evaporation of solvent, lyophilization of the residue and dissolution in DMSO, single tripeptoids of the general structure A-B-C were obtained in which all three positions were structurally defined.

Resynthesis of Single Tripeptoids

The resynthesis of individual tripeptoids was performed with 33 μmol of Fmoc Rink amide polystyrene resin (loading 0.48 mmol/g). After removal of the Fmoc protecting group, three cyles of bromoacetylation and nucleophilic displacement, the tripeptoids were cleaved from the resin, the solvents removed and the product lyophilized. The crude products were purified by HPLC, yielding pure tripeptoids. The identity of the tripeptoids was checked by analytical HPLC and MALDI mass spectrometry.

Synthesis of Tetrapeptoids

For the synthesis of tetrapeptoids for the GRP/bombesin receptor, the tripeptoid H-Gly(5)-Gly(14)-Gly(61)-NH_2 was prepared on Rink amide polystyrene resin using the semiautomatic instrument and the chemistry described above. After bromoacetylation of the tripeptoid, the resin was distributed in 69 equal portions and each portion was treated with one of the 69 different amines. The tetrapeptoids were individually cleaved from the resin and further purified as described above for the tripeptoids. The tetrapeptoids for the MSH receptor were prepared in the same way, starting from H-Gly(60)-Gly(21)-Gly(66)-resin.

Desamino-Gly(5)-Gly(60)-Gly(21)-Gly(66)-NH_2, which lacks the N-terminal amino group and which is a potential radiotracer for the MC1 receptor, was synthesized starting from resin-bound H-Gly(60)-Gly(21)-Gly (66)-NH_2 by treatment with a two-fold excess of 4-hydroxyphenylpropionic acid in the presence of equal molar amounts of hydroxybenzotriazol and diisopropylcarbodiimide. The desamino-tetrapeptoid was cleaved from the resin and purified by HPLC to yield the final product in >95% purity. This compound was used for radioiodination employing the equimolar chloramine T method [19] followed by a two-step purification scheme with a C_{18} cartridge and HPLC.

Cell Binding Assays

Human HBL melanoma cells and rat AR4-2J pancreatic tumor cells were maintained according to standard protocols [17, 18]. The preparation of [^{125}I]-

[Nle^4, D-Phe^7]-α-MSH and [^{125}I]-[Tyr^4]-bombesin radioligands has been described previously [18, 19]. The receptor binding assay was performed with the cells in suspension at 37°C (human melanoma cells) or 15°C (rat pancreatic tumor cells), either in polyethylene tubes or in 96-well microplates, to which appropriate protease inhibitors (MSH: 0.3 mM 1,10-phenanthroline; bombesin: 1 μM PMSF, 0.01% SBTI, 5 μg/ml leupeptin and 40 μg/ml bacitracin) had been added. Details of the assays can be found in Siegrist *et al.* [20] and, respectively, in Dietrich *et al.* [18]. Binding data were analyzed with the iterative program Ligand [21].

RESULTS AND DISCUSSION

Synthesis of the Peptoid Library

The goal of the preparation of this CCL was to obtain a peptoid library with a maximum diversity. To this end, 69 different commercially available primary amines were chosen in order to build up N-substituted glycine tri- and tetramers containing different kinds of side-chains such as unsubstituted and substituted aromatic and heterocyclic groups as well as allphatic, unsaturated (allylic and acetylenic) or negatively and positively charged groups. Hence, the tripeptoid library which consisted of 328,509 compounds, contained similar structural elements as an equivalent tripeptide library but exhibited about a 40-fold higher diversity.

The assembly of the peptoid library was adapted to standard solid-phase synthesis methods using a semi-automated instrument and a two-step approach of chain elongation: bromoacetic acid was first coupled to the resin and then the bromine displaced by a nucleophilic reaction using the 69 different primary amines. The Rink amide polystyrene resin [16] showed excellent performance in the chemistry applied and proved to be very stable against the different reaction conditions. After cleavage of the peptoids from the resin of the first ('original') library, 69 compound mixtures were obtained, each containing 4,761 peptoids. The deconvolution library was

prepared in the same way, and analysis by HPLC of the crude products from this library showed that the purity of the products after cleavage from the resin was very good. This again demonstrates the versatility of the Rink amide resin for this type of synthesis.

Resynthesis of individual tripeptoids (and tetrapeptoids) on the Rink amide resin yielded the compounds, after preparative HPLC purification, in 10-20% yield and with a purity of >95% (95-99%). Table 1 shows the analytical data of seven selected tripeptoids that displayed good binding activity to the MC1 or the GRP/bombesin receptor. MALDI mass spectrometry confirmed the molecular mass of all of these compounds, and therefore we can assume that the molecular identity of the biologically active compounds in the library mixture was the same. This is supported by the fact that the biological characteristics of the individually resynthesized compounds corresponded with the data obtained with the library mixtures.

Some of the tripeptoids were resynthesized as inverse sequence; for example, the tripeptoid H-Gly(66)-Gly(21)-Gly(60)-NH_2 (peptoid 5) was also prepared as H-Gly(60)-Gly(21)-Gly(66)-NH_2 (peptoid 1) with the positions of residues 60 and 66 exchanged (Table 1). Interestingly, the latter compound was about 4-fold more potent than the former (see also below).

Identification of MC1 and GRP/Bombesin Receptor Ligands

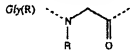
The 69 subpools of the initial library were tested in the two binding assays, (i) for MC1 receptor binding activity using melanoma cells and (ii) for GRP/bombesin receptor binding activity with pancreatic tumor cells. The sublibraries with the highest affinity to the MC1 receptor and, respectively, the GRP-preferring bombesin receptor were identified by an iterative process originally introduced by Houghten *et al.* [22]. Fig. 1 shows the deconvolution process for the biologically active tripeptoids with human HBL melanoma cells and [^{125}I]-[Nle^4, D-Phe^7]-α-MSH radioligand. The concentration of peptoid employed in the binding assay of the first two steps was 10 nM per

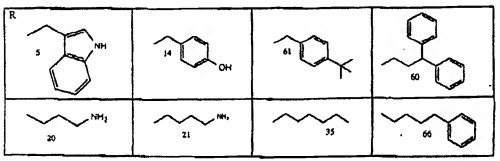
TABLE 1

Structure, receptor binding and analytical data of seven peptoid ligands after resynthesis, as tested in the MC1 receptor and the GRP/bombesin receptor assay.

Peptoid	Sequence[a]	K_D (mmol/l)[b] ± SD	MW (g/mol) MS (MH^+)[c]	Yield (mg)[d]	Retention time (min)[e]
1	H-Gly(60)-Gly(21)-Gly(66)-NH_2	1.58 ± 0.11	586.79 587.8	3.67 (19.0%)	12.41
2	H-Gly(60)-Gly(21)-Gly(61)-NH_2	1.93 ± 0.22	599.83 600.9	4.34 (21.9%)	13.15
3	H-Gly(60)-Gly(21)-Gly(60)-NH_2	1.99 ± 0.11	647.87 648.6	2.65 (12.4%)	12.27
4	H-Gly(60)-Gly(20)-Gly(60)-NH_2	2.07 ± 0.55	571.76 571.9	2.85 (15.1%)	12.41
5	H-Gly(66)-Gly(21)-Gly(60)-NH_2	4.06 ± 0.99	585.79 586.7	2.19 (11.3%)	12.29
6	H-Gly(60)-Gly(21)-Gly(35)-NH_2	6.48 ± 1.73	537.75 538.2	2.31 (13.0%)	11.91
7	H-Gly(5)-Gly(14)-Gly(61)-NH_2	3.40 ± 0.50	583.74 585.2	4.40 (22.8%)	10.72

[a] The monomeric building units correspond to the N-substituted glycines whose side-chains R (numbers in brackets) correspond to the structures shown below.
[b] K_D values were calculated from competition binding data with MSH radioligand and B16-F1 mouse melanoma cells (peptoids 1-6) and bombesin radioligand and rat AR4-2J pancreatic tumor cells (peptoid 7).
[c] Calculated molecular weights (MW) are listed together with the molecular mass found by MALDI mass spectrometry (MS).
[d] The yield reflects the recovery of pure product after RP-HPLC purification.
[e] Retention time of analytical RP-HPLC, as described in Materials and Methods.





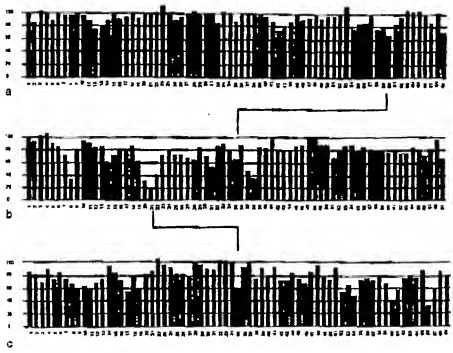


Fig. 1. *Identification of MSH receptor ligands using human HBL melanoma cells and [^{125}I]-[Nle^4, D-Phe^7]-α-MSH radioligand.* Specific binding (shown as % displacement) was determined for all 69 sublibraries of the original library (a) from which the most potent one was selected for resynthesis of the deconvolution library and testing of the second round of 69 sublibraries (b). The most potent sublibrary of (b) was selected for the preparation of 69 single compounds (c). For the binding experiments of (a), the concentration of the displacing peptoids was 10 nM per compound or 47.6 μM for the total of 4,761 compounds in each assay. For the experiments of (b), a 10-fold lower concentration for each peptoid mixture was used (4.7 μM for a total of 69 compounds or 69 nM for an individual compound). The single peptoids of experiment (c) were all tested at a concentration of 4.7 μM.

compound; a 10-fold lower concentration was also employed (not shown). For the binding assay of the last step, 1 nM concentrations per compound were used (Fig. 1) as well as 0.1 nM concentrations (not shown). The deconvolution for the peptoids with GRP/bombesin receptor binding activity was done in exactly the same way. About ten tripeptoids from each series were chosen for resynthesis and were individually evaluated for radioligand displacement at the MC1 receptor or, respectively, the GRP/bombesin receptor and the K_D values determined from competition binding experiments using at least 9 different concentrations of the displacing peptoids.

Binding Activities of MC1 Receptor Ligands

The K_D values of the tripeptoids most active at the MC1 receptor are shown in Table 1. Peptoids 2-6 originate from the deconvolution process and showed K_D values ranging from 1.93 to 6.48 mmol/l. The most potent compound, peptoid 1 (Fig. 2c), displayed a K_D of 1.58 mmol/l and represents peptoid 5 with the position of the terminal residues exchanged (see above). Peptoid 1 has a striking resemblance with the tripeptide H-Trp-Arg-Leu-NH_2 which was shown to be an MC1 receptor antagonist [14]. In general, all peptoids of this series share the following structural features: aromatic residue followed by a basic residue followed by an aromatic or aliphatic residue. This structural feature is also found in the active core of the α-MSH MC1 receptor ligands.

Extension of peptoid 1 by any of the 69 different amines at the N-terminus did not or only marginally increase the affinity to the MC1 receptor (not shown). On the other hand, addition of the 4-hydroxyphenylpropionyl residue to the N-terminal amino group of peptoid 1, thus forming a desamino-tetrapeptoid (Fig. 2d), increased the receptor binding activity by a factor of almost 2 (K_D = 850 nmol/l). In preliminary experiments, this compound was radioiodinated and studied in vitro and in vivo; however, the peptoid seemed to be too lipophilic for successful tumor targeting as the

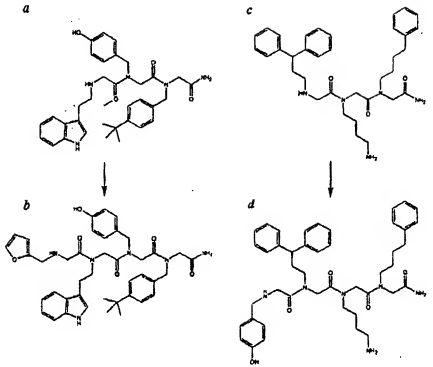


Fig. 2. *Structure of representative examples of tri- and tetrapeptoid ligands for the GRP/bombesin receptor and the MSH (MC1) receptor.* N-Terminal extension of H-Gly(5)-Gly(14)-Gly(61)-NH_2 (a) by one unit to H-Gly(2)-Gly(5)-Gly(14)-Gly(61)-NH_2 (b) led to a 12-fold higher affinity for the GRP/bombesin receptor whereas N-terminal extension of H-Gly(60)-Gly(21)-Gly(66)-NH_2 (c) by one unit to H-Gly(5)-Gly(60)-Gly(21)-Gly(66)-NH_2 (d) did not markedly change the affinity for the MSH (MC1) receptor but yielded a peptoid suitable for iodination.

uptake by the liver was higher than that observed with α-MSH peptides (data not shown). It is interesting to note that N- and C-terminal extension of peptoid 1 by those (or similar) amino acid residues found at the particular position within the α-MSH molecule, thus forming hybrid peptide-peptoid hexa- or nonamer structures, displayed binding affinities in the nanomolar range similar to the corresponding peptide; yet the biostability seemed to be increased as compared to that of the peptide (unpublished data).

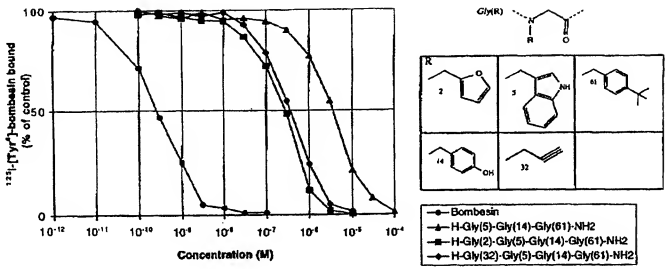


Fig. 3. *Competition binding analysis for bombesin, a tripeptoid and two tetrapeptoids using AR4-2J pancreatic tumor cells and [^{125}I]-[Tyr^4]-bombesin as radioligand.* The data are the mean of at least five determinations and represent percent specific binding of the radioligand in the presence of increasing concentrations of bombesin or peptoids. The side-chains R (number in brackets) of the N-substituted glycines correspond to the structures shown in the box.

Analysis of GRP/Bombesin Receptor Ligands

The tripeptoid most active at the GRP-preferring bombesin receptor was H-Gly(5)-Gly(14)-Gly(61)-NH_2 (Fig. 2a) which showed a K_D of 3.4 mmol/l (Table 1; peptoid 7). Extension by one residue at the N-terminus led to two tetrapeptoid structures with almost the same binding characteristics and which displayed an approx. 10-fold higher affinity for the GRP/bombesin receptor than the tripeptoid (Fig. 3). The more potent tetrapeptoid H-Gly(2)-Gly(5)-Gly(14)-Gly(61)-NH_2 (Fig. 2b) had a K_D value of 280 nmol/l. This was still about 1,000-fold lower than that of bombesin. However, as the biostability of this tetrapeptoid is expected to be much better than that of bombesin, also in *in vitro* assays, this compound may prove useful as radioligand for receptor assays. Preliminary data with the *in vitro* amylase-release bioassay showed that the GRP-preferring bombesin peptoids had mixed agonist and antagonist properties (data not shown). Whether these compounds are inverse agonists is not yet clear.

CONCLUSION

These results demonstrate that the testing of a peptoid library containing 328,509 single compounds led to the successful identification of new ligands for both the MSH (MC1) receptor as well as for the GRP-preferring bombesin receptor. Some of the tripeptoids displayed binding affinities in the low micromolar range and the tetrapeptoids in the nano- to micromolar range. These compounds have low molecular weights (<1,000) and are supposed to be more stable *in vitro* and *in vivo* than peptide ligands. Their activity is however lower than that of the natural ligands but may still be high enough for specific applications. The preliminary *in vivo* data with the MC1 receptor-specific desamino-tetrapeptoid indicate, however, that the structures need further modifications (e.g. reduction of the lipophilicity and aromaticity) in order to minimize the uptake by the liver. Nevertheless, peptoids or peptoid-peptide hybrids may prove useful for the *in vivo* application for tumor localization using ^{111}In- or ^{99m}Tc-labelled peptoids, or

for *in vitro* characterization of receptors in biological tissues or on cells which exhibit high protease activity leading to rapid degradation of peptide ligands.

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Synthesis, Chemical, Radiochemical and Radiobiological Evaluation of a New ^{99m}Tc-labelled Bombesin-like Peptide

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A new pentadecapeptide bombesin analogue was prepared by Fmoc synthesis, purified by HPLC and identified by electron ionization mass spectrometry. The biological activity of the new peptide was tested on isolated human colonic muscle cells and compared to native bombesin. Labelling of the new biomolecule with Tc-99m yielded a single radioactive species which remained stable at room temperature for eight hours. In a binding assay, the radiolabelled peptide showed high affinity for oat-cell carcinoma (K_d = 9.8 nM) and colorectal adenocarcinoma (K_d = 27.2 nM). Biodistribution studies, performed in normal rodents, indicated uptake by organs that normally express bombesin receptors, such as liver, intestines and kidneys. Scintigraphic studies, performed in nude mice transplanted with small cell lung carcinoma and colon cancer cells, showed significant tumor uptake two hours p.i. The new synthetic pentadecapeptide appears to have promise for several malignancies, including oat-cell lung carcinoma, colorectal cancer and gastroenteropancreatic (GEP) tumors.

INTRODUCTION

Small neuropeptides, labelled with gamma and/or beta emitting radionuclides, are currently being investigated for their ability to bind to cell-surface receptors, which are overexpressed in some malignant tissues.[1] These molecules are potentially useful for radionuclide detection and/or therapy of tumors. At the moment neuroendocrine tumors represent the most intriguing category of target tissues for radiolabelled peptides.[2] ^{111}In octreotide, a somatostatin analogue, has generated increasing interest for the diagnosis and localization of a number of endocrine tumors including tumors of the hypophysis, thyroid, adrenals, small cell lung carcinomas and neuroendocrine gastroenteropancreatic (GEP) tumors.[3–8] Several non endocrine carcinomas, such as lung adenocarcinoma[9] and breast cancer[10] have also shown enhanced uptake of ^{111}In-octreotide. Attention has also been focused on the amphibian peptide bombesin (BN), originally isolated from frog skin[7] and the molecularly related-gastrin releasing peptide (GRP). Overexpression of receptors for both BN and GRP has been encountered on the cell surface of several malignant tissues, particularly in the case of lung cancer[11–13] and colon cancer.[14,15] These peptides act as neurotransmitters and endocrine cancer cell growth factors.[16,17] In recent investigations modification of their structure has been attempted in order to obtain derivatives which might easily be labeled with radionuclides, suitable for use in imaging and therapy. Thus, ^{125}I-Tyr^4-BN is al-

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ready commercially available, while the meta-iodophenyl-Des-Met[14]-BN derivative (mIP-bombesin) has been synthesized and labelled with I-125 with good localizaion in an ovarian tumor model.[18] Also, some new bombesin analogues, containing a 6-carbon linker, have been prepared and labelled wih Rhenium-188, resulting in positive *in vitro* binding to prostate cancer cells.[19] More recent studies refer to Technetium-99m labelling of BN. Labelling was performed either directly, after attaching two diaminedithiol (DADT) groups to the BN sequence[20] or indirectly by coupling BN to a ^{99m}Tc-preformed dithiadiphosphine ligand.[21] Both types of conjugates are referred as having a high *in vitro* affinity for cells with bombesin receptors. However, though these types of conjugates appear to be promising, the chemistry for their preparation is complicated and time-consuming.

The aim of the present study was to design and synthesize a BN analogue that could be easily labelled with Tc. Thus, the following pentadecapeptide, bombesin analogue, has been synthesized, radiolabelled and investigated[22]:

Cys-*Aca*-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2,

where *Aca* represents 6-amino-n-hexanoic acid.

MATERIALS AND METHODS

Synthesis of the peptide amide was carried out manually by using α-fluorenylmethoxycarbonyl (Fmoc) amino acids, onto Rink Amide resin[23] following the method of Fmoc solid phase peptide synthesis.[24] Glutamine, asparagine and tryptophan were used unprotected in their side chain, Fmoc-Gln-OH, Fmoc-Asp-OH and Fmoc-Trp-OH. The side chain functional group of cysteine and histidine were protected as trityl thioether, Fmoc-Cys(Trt)-OH and Fmoc-His(Trt)-OH and of arginine as the 2,2,5,7,8 pente-methylchromane-6-sulfonyl derivative, Fmoc-Arg(Pmc)-OH. Couplings were performed by using DIPC/HOBt in DimethylFormAmide (DMF). Coupling success was checked by the established Kaiser ninhydrin test.[25] Deprotection of the Fmoc group was achieved by repetitive treatment with 20% piperidine in DMF. The resin-bound peptide was removed from the resin and amino acid side chains were deprotected by treatment with a cocktail of Trifluoro Acetic Acid (TFA), CH_2Cl_2, water and scavengers. After removing the organic solvents, the crude product was precipitated with cold diethyl ether.

The crude peptide was dissolved in water and purified by semi-preparative reverse phase high-performance liquid chromatography (HPLC) on a Prep Nova-Pak HR-C18 column (Waters). Elution was performed by a gradient system consisting of two solvents: A: 0.05% TFA in water and B: 60% Acetonitrile (ACN) in solution A at a 1.3 ml/min flow rate. Detection of the peptide was achieved with a variable-multi-wavelength detector set at 220 nm. The overall yield of the synthesis was 40%.

The amino acid content of the synthetic peptide was confirmed by amino acid analysis. The purified peptide was hydrolyzed with 6N HCl for 1 hour, at 150°C. The acid hydrolysate was then derivatized with phenyl isothiocyanate (PITC), according to an established protocol proposed by Waters. The derivatized product was analyzed with a PICO-TAG amino acid analysis system (Waters) and compared to amino acid standards.

For the electrospray ionization mass spectrometry (ESI-MS) analysis, test peptide solution of 0.5 mg/ml in 50% acetonitrile/water as eluent containing 1% acetic acid was infused at a flow-rate of 3 μl/min, using a Harvant syringe pump, into an electrospray interface mass spectrometer (Micromass Platform II). In the electrospray source, the spray needle was grounded: voltages of −4.5, −3.5, −3.0 kV were applied to the capillary, to the end plate and to the cylindrical electrodes, respectively. The capillary/skimmer potential difference was set at 150 V; the other source lenses were held at potentials that optimized the signal intensity. Hot nitrogen gas was used for desolvation. According to this gentle ionization method[26] analyte ions were formed, often bearing multiple charges. The charge on each ion and the molecular mass of the peptide were determined by deconvolution algorithms.

Biological Evaluation

Smooth muscle cells from different species including humans, express specific BN receptors. BN has also been reported to always induce a contractile response in smooth muscle cell preparations from different gastrointestinal tracts.[27,28] Thus the biological efficacy of the new BN analogue was measured on human colonic smooth muscle cells by comparing its contractile activity to that of native bombesin. Preparation of dis-

persed smooth muscle cells from human colon has already been described in detail.[29] Briefly, 2.5 cm long specimens of whole colon were obtained with the prior consent from patients undergoing surgery for cancer of the colon-rectum. The segment of intestine taken was from an unobstructed region of the bowel which was farthest away from the tumoral lesion. Immediately after excision, segments from the intertaenial regions were dissected and placed in a standard ice-cold incubation solution bubbled with O_2. Mucosa and submucosa were removed with scissors and 0.5 mm thick slices of circular muscle were obtained by the use of a Stadie-Riggs tissue slicer (Thomas Scientific Apparatus, Philadelphia, PA, USA). Muscle strips were incubated at 31°C for two 60 min periods in 15 ml of a standard incubation solution containing 0.1% collagenase (150 U/ml). At the end of the second incubation period, the partly digested strips were minced, washed with 50 ml of a collagenase-free standard incubation solution and resuspended in 15 ml of fresh standard solution. Smooth muscle cells were allowed to disperse spontaneously for 30 min and were then harvested by filtration through a 500 μm Nitex mesh.

The contractile response was measured using 0.5 ml of a standard solution containing colonic smooth muscle cells (10^4 cells/ml) which had been incubated with the agents to be tested. The reaction was stopped by the addition of acrolein (final concentration 1% v/v). The muscle cell concentration was measured by image scanning micrometry (Lasico, Los Angeles, CA, USA). The length of 50 muscle cells, randomly encountered in successive microscopic fields, was measured. Contraction was expressed as percent decrease in the length of cells incubated for 30 sec with contracting agents, with respect to the length of the untreated cells. Results were expressed as mean ± SE of three experiments.

^{99m}Tc-Labelling

For the preparation of the labelled product, sodium gluconate was used as an intermediate exchange ligand for Technetium-99m; pertechnetate was reduced by stannous ions.[30] Thus, a solid mixture containing 1.0 g (4.584 mmol) sodium gluconate, 2.0 g (23.807 mmol) sodium bicarbonate and 0.015 g (0.791 mmol) stannous chloride was homogenized and kept dry. A quantity of 0.003 g of the above mixture was dissolved in 1.0 ml of a sodium pertechnetate solution, containing 370-555 MBq (10.0-15.0 mCi) of Tc-99m. An aliquot of 0.2 ml of the above solution was added to 1.8 ml of a 10% alcohol solution, containing 0.3 mg (0.174 mmol) of the peptide under study. The mixture was left at room temperature and the exchange reaction was completed in ten minutes. The labelling efficiency and the *in vitro* stability of the radiolabelled product were determined by analytical High Performance Liquid Chromatography (HPLC) with a Waters C_{18} reverse phase column μ-Bondapack C_{18}, (3.9 mm I.D. × 300 mm). For the elution a linear gradient system, consisting of A: 0.05% TFA in water and B: 60% ACN in solvent A was used. Eluent was passed through the UV detector, at 220 nm and through a sodium iodide scintillation detector, connected both to a computer for data analysis and storage.

Stability study of the radiolabelled derivative: In vitro stability was studied in the presence of cysteine and by incubation with human serum at different temperatures according to already published methods.[33]

Cancer Cell Binding Assay

Cell binding assays were performed using the epithelial-like Colo 205 cell line (CCL 222 of the American Type Culture Collection) isolated from human colon adenocarcinoma,[31] and the AE1 cells isolated from human pulmonary oat-cells carcinoma. The cancer cells of the Colo 205 and the AE1 cell lines were grown as monolayers and suspension cultures respectively, in RPMI medium supplemented with 10% fetal bovine serum at 37°C and 5% CO_2. Subculturing of the Colo 205 monolayers were performed after trypsinization (0.25% trypsin in EBSS) using a 1:2 split ratio every 2–3 days,[32] whereas the AE1 suspension cultures were subcultured every 5–7 days by centrifugation and resuspension of cells in complete culture medium using a 1:2 split ratio. For the binding assays, the cells were harvested, washed twice with PBS and resuspended in PBS containing 1% BSA at a cell density of 10^6 cells/100 μl. All the work, except the incubations—which were performed at room temperature for one hour[33]—was carried out on ice. Six dilutions of the labelled peptide were used. The incubation mixtures consisted of 10^6 cells plus labelled peptide at concentrations of 730, 73, 51, 36.5, 22 and 7.3 nM in PBS containing 1% BSA. All samples were assayed in duplicate.

After incubation the samples were centrifuged. The resulting supernatants were separately col-

lected from each eppendorf and placed into tubes for counting. The pellets were resuspended in 300 μl PBS and transferred onto 0.22 m filters (Millipore), which were placed into the wells of a manifold filtration apparatus (12 wells/apparatus). The samples were vacuum dried onto the filters. The filters were washed four times with PBS and dried in the same manner as that described above. In continuation, the filters were placed into counting tubes and the radioactivity of the filters, as well as that of the first supernatants, were measured in a gamma counter (minaxi autogamma 5000 series, Packard). The results were assessed using the Scatchard analysis.[34,35]

Biodistribution Studies in Normal Rodents

The *in vivo* behavior of the new Tc-99m labelled peptide was initially studied in normal female Swiss mice (average weight 25 g) and in female Wistar rats (average weight 120 g) by intravenous administration into the tail vein. Animals were sacrificed at predetermined time intervals and the main organs, as well as blood, muscle and urine samples were removed, weighed and counted. The percentage of the injected dose per organ was calculated, in comparison to a standard. *In vivo* studies were performed in compliance with the European legislation. Animal protocols have been approved by the Hellenic authorities.

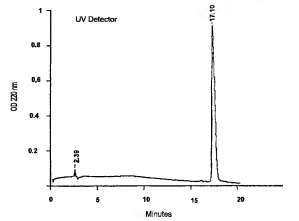

Figure 1. HPLC profile of the synthetic BN-derivative

Tumor Imaging

Tumor localization was evaluated in female, BALB/c nude mice, 5 weeks old. Animals were housed in an isolated, pathogen-free and temperature controlled environment. They were fed *ad libitum* with sterilized food and autoclaved water. Suspensions containing 10^7 cells of each cell line under study in 0.5 ml saline were injected subcutaneously into the right thigh of each animal. Three weeks later the tumors developed could easily be distinguished. A quantity of 0.1

Table 1. Amino acid analysis of the new Bombesin analogue

Amino acid	Theoretical composition	Amino acid content obtained
Asx	1	1.062
Glx	2	2.071
Gly	2	2.090
His	1	1.007
Arg	1	1.043
Ala	1	1.073
Hex	1	0.924
Val	1	0.996
Met	1	1.020
Cys	1	0.773
Leu	2	1.963

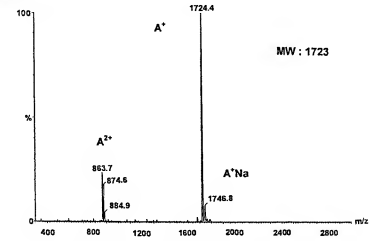

Figure 2. ESI mass spectra of the synthetic molecule

ml (37MBq/ml) of the radiopharmaceutical under study was injected intravenously into the tail vein of the animals, which were sacrificed with a lethal dose of diazepam two hours p.i. Images were obtained at a Sopha γ-camera, using a pinhole collimator. Followingly the tumor was excised along with a muscle sample of the respective point of the opposite leg and the tumor to non-tumor ratio was calculated. The uptake of the radiolabelled peptide in the other organs of the tumor-bearing animals was determined as described for normal mice.

RESULTS

The BN-like peptide was prepared by Fmoc solid phase synthesis with an overall yield of 40%. It was purified by RP-HPLC, yielding a highly pure final product, as characterized by analytical RP-HPLC. The elution profile of the synthetic bombesin derivative is presented in Fig. 1. The new biomolecule was eluted at 17.10 min as a single peak.

The amino acid analysis revealed perfect agreement with the expected amino acid content of the peptide (Table 1).

The ESI mass spectra of the synthetic molecule (Fig. 2) has two major peaks at m/z 1724.4 and 874.7 corresponding to the protonated molecule (charge +1) and the molecule bearing a proton and an alkali cation (charge +2). Other related minor ions, $[M + Na]^+$, $[M + 2H]^{2+}$, were also present.

The molecular mass (1723.4) of the synthetic peptide obtained from the mass analysis was identical to the average molecular mass calculated (1723) based on the peptide's primary structure.

Table 2. Ability of new Bombesin analogue to induce contraction of human colonic circular smooth muscle cells

Agent	Cell Length (μm)
None	91.1±1.2
Carbachol (30 nM)	72.0±0.8*
Native Bombesin (1nM)	74.4±1.0*
New Bombesin (1nM)	75.0±0.5*

**Each value is the mean ± SD of at least three experiments. P<0.01 vs. control*

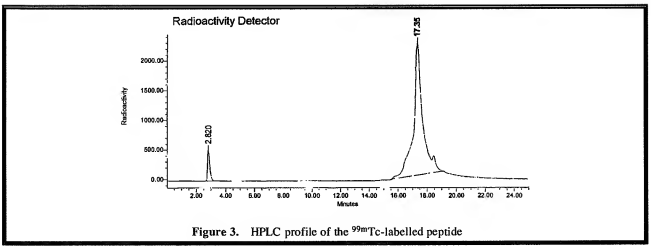

Figure 3. HPLC profile of the ^{99m}Tc-labelled peptide

At a 1 nM concentration, the synthetic peptide induced 18% shortening of colonic smooth muscle. Also 1 nM of the native peptide and 30 nM of the reference contracting agent, carbachol, induced an 18% decrease in cell length (Table 2) in the control experiments.

Technetium-99m labelling was easily performed at room temperature by an exchange reaction with preformed ^{99m}Tc-gluconate. High Performance Liquid Chromatography of the ^{99m}Tc-labelled derivative indicated the formation of a single radioactive species with a retention time of 17.0–17.5 min (Fig. 3), at a $\geq$ 97% yield and with a specific activity of 5–10 mCi/mg. The recovery of the HPLC column was higher than 90%, indicating the absence of higher molecular weight Technetium-99m species. The radiolabelled derivative remained stable for up to 8.0 hrs when kept in refrigerator.

The cysteine challenge experiments indicated that about 50% of the label are lost by transchelation to cysteine at 10:1 molar excess of cysteine, during 1 h incubation at room temperature. At 100:1 and 1000:1 molar excess, the values are 80% and 87% respectively. Plasma stability studies are in accordance to the cysteine challenge results.

Scatchard analysis of the data obtained from the binding assay experiments of the technetium-labelled new peptide to the binding sites of the Colo 205 human adenocarcinoma and the oat-cell carcinoma cell lines revealed an apparent Kd of 27.2 $\pm$ 8.14 nM and 9.8 $\pm$ 2.03 nM respectively. This finding is indicative of a high to moderately high affinity amongst the ligand and the binding sites of the colon cancer cell line (Fig. 4) and a high affinity with the pulmonary neuro-endocrine tumor (Fig. 5).

The biodistribution of the labelled peptide was evaluated in mice. Results are reported in Table 3.

The new ^{99m}Tc-labelled peptide presented relatively fast blood clearance. Thus, in this species, the blood value was 2.39% dose per organ two hours after administration and remained at that level (2.08%) for up to six hours p.i. Intestinal values were stable for the same time period, ranging from 28.07% two hours p.i. up to 29.91% for the six-hour time interval. Kidney values were also stable, ranging from 17.75% two hours after administration to 12.77% six hours p.i.

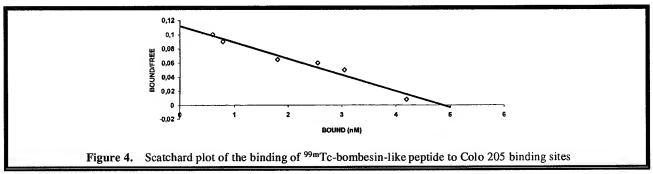

Figure 4. Scatchard plot of the binding of ^{99m}Tc-bombesin-like peptide to Colo 205 binding sites

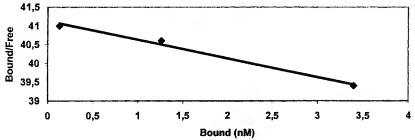

Figure 5. Binding of the ^{99m}Tc-labelled Bombesine derivative on Small Cell Lung Carcinoma Cells AE1

The biodistribution was also studied in rats for the 4.0 and 24.0 hours time points (Table 4).

Results indicated similarly high gastrointestinal uptake up to 24 hours p.i. Thus, the uptake in the liver, expressed as percent of the dose injected per organ, was 15.84%, twenty four hours p.i., while the respective intestinal value was 5.90% for the same time interval. Gastric values were low in both species, indicating the absence of free pertechnetate and advocating for ***in vivo*** stability.

In Vivo Tumor Uptake

Characteristic images of nude mice, transplanted with: (A) colon cancer cells and (B) small cell lung cancer cells are presented in Fig. 6. In both cases the experimentally induced tumors were clearly delineated. Biodistribution studies shows that the average of tumor to normal tissue ratio was about 3.5 two hrs p.i., for the colon cancer bearing animals and 7.8 for the animals transplanted with the small cell lung cancer line. In the rest of the organs, peptide uptake was similar to that of the normal animals.

DISCUSSION

A new bombesin analogue has been synthesized and studied. This biomolecule was designed to be suitable for Tc or Re labelling: thus only the N-terminal end of BN was changed. The structure of the peptide under study is:

Cys-Aca-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2

where Aca represents: 6-amino-n-hexanoic acid
The native bombesin structure is:

pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2.

Table 3. Biodistribution in Mice*

(% Injected Dose per Organ ± SD)

	2.0 hrs	4.0 hrs	6.0 hrs	24.0 hrs
Blood	2.39±0.4	2.47 ±0.6	2.08 ±0.4	0.26 ±0.1
Liver	37.53 ±2.4	19.15 ±6.9	14.84 ±2.5	5.86 ±1.7
Kidneys	17.75 ±4.1	10.45 ±2.9	12.77 ± 2.3	3.45 ±1.1
Stomach	2.45 ±0.4	1.58 ±0.4	1.97 ± 0.4	0.33 ±0.1
Intestines	28.07 ±2.2	29.87 ±9.9	27.91 ± 10.5	1.80 ±0.7
Spleen	0.96 ±0.6	0.38±0.2	0.63 ± 0.2	0.30 ±0.1
Lungs	1.95 ±0.1	1.03 ±0.6	0.72 ± 0.2	0.40 ±0.1
Pancreas	0.62 ±0.2	0.27 ±0.1	0.48 ± 0.2	0.08 ±0.1

**Each value is the average of five to seven animals*

Table 4. Biodistribution in Rats*

(% Injected Dose per Organ ± SD)

	4.0 hrs	24.0 hrs
Blood	1.84±0.4	0.55±0.0
Liver	11.25±5.9	15.84±0.3
Kidneys	15.03±1.0	9.92±1.8
Stomach	0.37±0.2	0.39±0.1
Intestines	19.26±6.4	5.90±2.2
Spleen	0.22±0.1	0.29±0.2
Lungs	1.02±0.8	0.64±0.5
Pancreas	0.11±0.0	0.04±0.0

**Each value is the average of five animals*

Comparing the structure of the new derivative to that of bombesin, it can be observed that a cysteine residue attached to a 6-amino-n-hexanoic acid (ACA) spacer arm has replaced pyroglutamic acid. In this way, we have kept the labelling point away from the receptor binding site of the molecule, located at the C-terminal. The bombesin analogue is a pentadecapeptide with a molecular weight of 1723. A single molecular species was found by the analytical methods applied. When lyophilized, the new molecule was found to be stable for the duration of the study period (six months). The contractile response of the BN-like peptide was estimated on colonic smooth muscle cells, in comparison to that of native bombesin. Incubation of the peptide with muscle cells showed that its biological activity was indistinguishable from that of native bombesin.

Labelling with Tc-99m was performed with high yield. The labelling protocol leads to the formation of a single radioactive peak, as indicated by the HPLC profile. The retention time of this peak is different from that of pertechnetate and gluconate, which in chromatographic system used, are eluted with the solvent front.

The biodistribution of the radiolabelled derivative in normal rodents shows significant retention into the liver, intestines and kidneys, as expected for any BN analogue and as reported by other investigators.[18,21] The ^{99m}Tc-labelled derivative was found to be stable for at least 8.0 h post labelling at 4°C. This means that the new peptide, labelled with Tc-99m, is promising for use as a diagnostic, tumor-seeking and/or therapeutic agent.

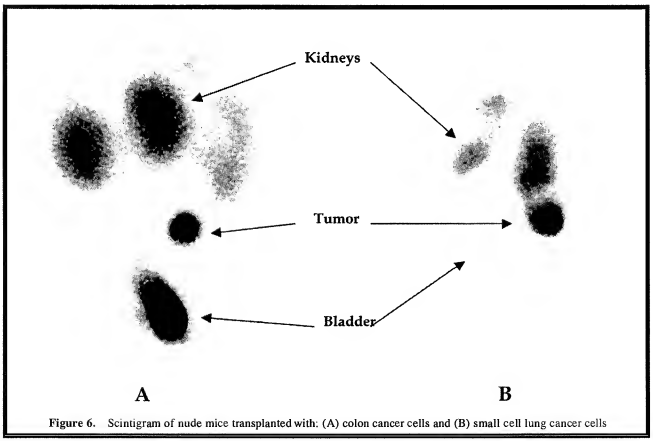

Figure 6. Scintigram of nude mice transplanted with: (A) colon cancer cells and (B) small cell lung cancer cells

The *in vivo* behavior of the labelled species is satisfactory, although the *in vitro* stability as determined experimentally by the cysteine challenge and serum stability studies can not be considered high.

The results of the binding assay, using the oat cell carcinoma, were the expected ones and concur with the findings of other workers. Moody et al.[12] and more recently Mahmond et al.[13] have shown that small cell lung carcinomas have a very high affinity for the bombesin/gastrin releasing peptide and that bombesin analogues effectively inhibit the growth of small cell lung carcinoma. Moreover, the moderately high affinity with colon cancer was an interesting result that ties in with the findings of Preston et al.[14] They found that several colon cancers expressed high affinity to bombesin binding sites of the gastrin-releasing peptide subtype. At present we can only speculate as to the significance of BN binding site expression on human colon cancers. However, these results warrant further study, since they indicate that bombesin-like peptides may also have a role in the pathogenesis of colon cancers and that bombesin receptor analogues may be of value in the treatment of these tumors as well.

Scintigraphic studies performed in nude mice, transplanted with colon cancer cells and small cell lung carcinoma cells, demonstrated significant tumor uptake. Although more conventional "blocking studies" are often used to demonstrate specific uptake, our scintigraphic imaging results in experimental animals have been considered indicative enough to characterize tumor localization as specific.

In conclusion, the experimental results presented in this work describe the synthesis and ^{99m}Tc-labelling of a new stable bombesin analogue with chemical and biological properties similar to those of native bombesin and with very promising properties as a new specific tumor-seeking radiopharmaceutical. Investigation of the structure of the Tc-99m complex through the respective non-radioactive rhenium complex is in progress. Also, additional cell culture studies using other cancer cell lines and further *in vivo* experiments are programmed so as to characterize fully this new bombesin analogue and provide further support for its possible future clinical use.

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Design, synthesis, and *in vitro* evaluation of cytotoxic analogs of bombesin-like peptides containing doxorubicin or its intensely potent derivative, 2-pyrrolinodoxorubicin

(targeted chemotherapeutic agents/hybrid molecules/receptor binding/antiproliferative activity)

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ABSTRACT **Five peptide fragments, based on the C-terminal sequence of bombesin (BN)-(6-14) or BN-(7-14), were selected as carriers for radicals doxorubicin (DOX) and 2-pyrrolino-DOX to create hybrid cytotoxic analogs. All these compounds had a reduced peptide bond (CH_2-NH or CH_2-N) between positions 13 (Phe or Leu) and 14 (Phe, Leu, or Tac) (Tac = thiazolidine-4-carboxylic acid). Three pseudononapeptide carriers contained N-terminal D-Phe or D-Tpi at position 6 (Tpi = 2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid). Two pseudooctapeptides had Gln^7 at the N terminus. The conjugation of *N*-(9-fluorenylmethoxycarbonyl)doxorubicin (*N*-Fmoc-DOX)-14-*O*-hemiglutarate to the peptide carriers at the N terminus resulted in cytotoxic hybrids of BN-like peptides containing DOX. These hybrids could then be converted to analogs with 2-pyrrolino-DOX by a reaction with 4-iodobutyraldehyde. The ability of the carriers and the conjugates to inhibit the binding of ^{125}I-labeled [Tyr^4]BN to receptors for BN/gastrin releasing peptide (GRP) on Swiss 3T3 cells was determined. Cytotoxic conjugates of pseudooctapeptide carrier analogs displayed the highest binding affinity (K_D ≈1 nM). The cytotoxic BN analogs and their corresponding cytotoxic radicals exerted similar inhibitory effects on the *in vitro* growth of CFPAC-1 human pancreatic cancer, DMS-53 human lung cancer, PC-3 human prostate cancer, and MKN-45 human gastric cancer cell lines that have receptors for BN/GRP. In DMS-53 cells, the activity of 2-pyrrolino-DOX and its conjugates was ≈2500 times higher than that of DOX and its hybrids. These highly potent cytotoxic analogs of BN have been designed as targeted anti-tumor agents for the treatment of various cancers that possess receptors for BN/GRP.**

Following the isolation of the tetradecapeptide bombesin (BN) from frog skin, a variety of its amphibian and mammalian homologs has been isolated and identified (1). This family of BN-like peptides includes gastrin-releasing peptide (GRP), a 27-amino acid peptide, which was considered to be the mammalian counterpart of BN, the amphibian ranatensin and neuromedin B, which was found in mammals, as well as the less known class of phyllolitorins (1). All these peptides have a highly conserved C terminus that is responsible for a wide variety of pharmacological effects, ranging from the release of gastrointestinal hormones to the effects on blood pressure, body temperature, and cardiac output (1). Various studies indicate that these peptides act as neuroregulatory hormones and growth factors in normal and neoplastic tissues (2) and exert their effects through binding to multiple receptors for the BN-like peptides. The receptors are located mainly in the central nervous system, in the digestive tract, and in other target organs such as the lung (2). Thus far, four different receptor subtypes for the BN family have been cloned and characterized. One of these receptors, found in large numbers (≈100,000 per cell) on the membranes of Swiss 3T3 murine fibroblasts, binds BN and GRP with a K_D in the nanomolar range (1). Another receptor subtype binds peptides of the neuromedin B family with an affinity ≈100 times higher than those peptides that have a C terminal in common with BN and GRP (1). A third subtype shows a 100 times lower affinity for BN and neuromedin B than the receptors specific for these peptides (1, 3). The fourth known receptor subtype was characterized as having a higher affinity for BN than for GRP (4).

During the past decade, extensive evidence has been gathered on the involvement of peptides of the BN family in the mitogenesis of various tumor cells including small cell lung carcinoma (SCLC) (5, 6), cancers of the gastrointestinal tract such as pancreatic cancer (7), colon cancer (8), as well as breast cancer (9). The putative role of BN-like peptides as autocrine growth factors for these tumors (5–9) prompted researchers to design and synthesize antagonists of BN and GRP in hope of finding a different approach for the treatment of certain cancers (10–16). Over the past few years, we have developed a series of powerful BN antagonists (12–16). One of these antagonists (RC-3095 = B_1, Table 1) showed promising tumor inhibitory effects in various animal cancer models and in nude mice bearing xenografts of human cancer cell lines and is presently undergoing clinical trials. Antitumoral effects of BN/GRP antagonists *in vivo* have been demonstrated on CFPAC-1 and SW-1990 human pancreatic cancers (17, 18), nitrosamine-induced pancreatic cancers in hamsters (19), H69 human SCLC (20), MKN45 and Hs746T human gastric cancers (21, 22), HT-29 human colon cancers (23, 24), PC-82, PC-3, and DU-145 human prostate cancers (25, 26), androgen independent Dunning R-3327-AT-1 rat prostate cancers (27), estrogen dependent and independent MXT mouse mammary cancers (28), MCF-7 MIII human breast cancer (29), and U-87MG and U-373MG human glioblastomas (30). Receptor analyses of these tumors showed the presence of high-affinity binding sites for ^{125}I[Tyr^4]BN (1, 2, 17–33). Recently, we described the synthesis and evaluation of cytotoxic analogs of luteinizing hormone-releasing hormone containing doxorubicin (DOX) or 2-pyrrolino-DOX, a derivative 500-1000 times more potent (34, 35). These cytotoxic analogs were developed for therapy of cancers that contain receptors for luteinizing

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Abbreviations: DOX, doxorubicin; BN, bombesin; Tpi, 2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole-3-carboxylic acid; GRP, gastrin-releasing peptide; SCLC, small cell lung carcinoma.

Table 1. Structures of cytotoxic BN analogs and carriers and their ability to displace [^{125}I-Tyr^4]BN binding to BN/GRP receptors on Swiss 3T3 cells

Structure	Receptor binding K_i,* nM
B_1 = [D-Tpi^{6,13} ψ^{14}, CH_2-NH, Leu^{14}]BN-(6-14)	3.5
AN-253 = DOX-14-*O*-glt-B_1	8.0
AN-254 = 2-pyrrolino-DOX-14-*O*-glt-B_1	13.0
B_2 = [D-Phe^{6,13} ψ^{14}, CH_2-NH, Phe^{14}]BN-(6-14)	4.3
AN-246 = DOX-14-*O*-glt-B_2	4.9
AN-247 = 2-pyrrolino-DOX-14-*O*-glt-B_2	8.6
B_3 = [D-Phe^{6,13} ψ^{14}, CH_2-N, Tac^{14}]BN-(6-14)	2.7
AN-161 = DOX-14-*O*-glt-B_3	2.9
AN-257 = 2-pyrrolino-DOX-14-*O*-glt-B_3	3.4
B_4 = [^{13} ψ^{14}, CH_2-NH, Leu^{14}]BN-(7-14)	>1000
AN-160 = DOX-14-*O*-glt-B_4	0.95
AN-215 = 2-pyrrolino-DOX-14-*O*-glt-B_4	1.6
B_5 = [^{13} ψ^{14}, CH_2-N, Tac^{14}]BN-(7-14)	>1000
AN-251 = DOX-14-*O*-glt-B_5	0.7
AN-252 = 2-pyrrolino-DOX-14-*O*-glt-B_5	0.6
BN = pGlu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2	2.0†

Tac, thiazolidine-4-carboxylic acid; Tpi, 2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]-indole-3-carboxylic acid

*Varying amounts of unlabeled peptide were used to determine the ability to displace [^{125}I-Tyr^4]BN binding; mean values of two or more independent tests (each performed in triplicate) are indicated (14).

†Mean value of 12 independent tests.

hormone-releasing hormone (35). The presence of receptors for BN-like peptides on a wide variety of tumors (17–33), prompted us to use some of our powerful BN/GRP antagonists as carrier molecules for targeting cytotoxic agents to tumor cells.

In this paper we report the design, synthesis, and biological evaluation *in vitro* of cytotoxic BN analogs containing DOX and 2-pyrrolino-DOX (34, 35). The tests *in vitro* included the determination of the binding affinities to BN/GRP receptors on Swiss 3T3 murine fibroblasts and of the cytotoxic activities on CFPAC-1 human pancreatic cancer, DMS-53 human lung cancer, PC-3 human prostate cancer, and MKN-45 human gastric cancer cell lines.

MATERIALS AND METHODS

Synthesis. Pseudononapeptide and pseudooctapeptide BN-like peptide carriers were synthesized as described (12–16). Cytotoxic conjugates of these peptides with DOX or 2-pyrrolino-DOX were prepared by an improvement of the procedure reported earlier for the formation of cytotoxic luteinizing hormone-releasing hormone conjugates (35).

Preparation of *N*-(9-fluorenylmethoxycarbonyl)-(*N*-Fmoc)-DOX-14-*O*-hemiglutarate. *N*-Fmoc-DOX (35) (1.3 g, 1.7 mmol) was dissolved in 15 ml of anhydrous pyridine and 50 ml of *N*,*N*-dimethylformamide (DMF) was added. The pyridine was then evaporated *in vacuo*, the DMF solution was concentrated to 30 ml to eliminate traces of water, and glutaric anhydride (750 mg, 6.6 mmol) was added followed by *N*,*N*-diisopropylethylamine (592 μl, 3.4 mmol). After 4 hr the reaction mixture contained ≈75% of the desired end product. The DMF solution was then poured into 500 ml of 5% aqueous acetic acid (AcOH) (vol/vol) on an ice bath. The precipitate formed was filtered off and washed three times with 200 ml of distilled water. After drying in a desiccator, the 1.45 g crude solid was dissolved in 10 ml of $CHCl_3$/AcOH (4:1, vol/vol) and applied on a column (2.5 × 30 cm), packed with 75 g of silicagel (Merck grade 9385; 230–400 mesh; pore size, 60 Å) equilibrated with $CHCl_3$/AcOH (4:1, vol/vol). Flash chromatography using this solvent system resulted in good separation of the desired end product. On TLC aluminum sheets precoated with silicagel 60 F_{254} (Merck Art No. 5554), using $CHCl_3$/AcOH (4:1, vol/vol) as eluent, the desired end product shows an R_f = 0.7, whereas the unreacted starting material runs at R_f = 0.5 and the diester derivative at R_f = 0.85. After combining the fractions containing pure material, the $CHCl_3$ was evaporated and the AcOH was concentrated to 30 ml. This solution was poured into 200 ml of water on an ice bath. The resulting precipitate was filtered off and washed three times with 200 ml of water. After drying in a desiccator, 950 mg of 98% pure *N*-Fmoc-DOX-14-*O*-hemiglutarate was obtained, representing a 56% overall yield, starting from 1 g of DOX × HCl.

This pure *N*-Fmoc-DOX-14-*O*-hemiglutarate was used for the preparation of cytotoxic conjugates of BN containing DOX, with yields higher than 60% (35). Cytotoxic BN analogs with DOX were converted to their 2-pyrrolino-DOX derivatives by a reaction with a 30-fold excess of 4-iodobutyraldehyde in DMF (35).

Analytical HPLC. A Beckman analytical HPLC system equipped with model 168 diode array detector and System Gold chromatography software (Beckman) was used to monitor the chemical reactions and check the purity. The column used was a Dynamax C_{18} (250 × 4.6 mm; pore size, 300 Å; particle size, 12 μm).

Purification. Final purification of all peptide conjugates was carried out on a Beckman model 342 semipreparative HPLC system, using an Aquapore Octyl (250 × 10 mm; pore size, 300 Å; particle size, 15 μm) column. The solvent system consisted of two components—0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 70% aqueous acetonitrile—and was used in linear gradient mode.

Analysis. Electrospray mass spectrometer Finnigan-MAT TSQ 7000 was used for the structural identification of the peptide conjugates.

Receptor Binding. Binding affinities of the analogs to receptors for BN/GRP on Swiss 3T3 cells were determined as described (14, 16, 32).

Cytotoxicity Assay. CFPAC-1 human pancreatic cancer, DMS-53 human SCLC, PC-3 human prostate cancer, and MKN-45 human gastric cancer cell lines were obtained from the American Type Culture Collection. These cells were cultured in media indicated in the footnotes to Tables 2 and 3. DOX, 2-pyrrolino-DOX and the cytotoxic BN/GRP analogs were dissolved in culture media and added at three different concentrations, as shown in detail in Tables 2 and 3. The determination of the cytotoxic activity of the analogs on all four cell lines was performed by using a colorimetric cytotoxicity assay in microtitration plates based on quantification of biomass by staining cells with crystal violet (36).

RESULTS

Design and Synthesis. To create targeted cytotoxic analogs of BN/GRP with specific high-affinity binding to BN/GRP receptors, three pseudononapeptide BN antagonists and two pseudooctapeptide BN-like peptides were selected as carriers. The chemical structures of these carriers were based on the C-terminal sequence of BN (Table 1). To form cytotoxic analogs containing DOX, the peptides were acylated at their N terminus with *N*-Fmoc-DOX-14-*O*-hemiglutarate as described (35). In our previous effort to synthesize cytotoxic analogs of luteinizing hormone-releasing hormone containing DOX, *N*-Fmoc-DOX-14-*O*-hemiglutarate was used in a crude form (35). In this study, we used an effective purification procedure based on silica-gel chromatography with $CHCl_3$/AcOH (4:1, vol/vol) as eluent for the separation of *N*-Fmoc-DOX-14-*O*-hemiglutarate from impurities such as *N*-Fmoc-

Table 2. Inhibitory effects of DOX, 2-pyrrolino-DOX (AN-201), and their conjugates with BN-like peptide carriers on the growth of CFPAC-1 human pancreatic cancer cell line and on DMS-53 human lung cancer cell line *in vitro*

Compound	Cell line	T/C Value						
		3×10^{-11} M	10^{-10} M	3×10^{-10} M	10^{-9} M	3×10^{-8} M	10^{-7} M	3×10^{-7} M
				Analogs with DOX				
AN-253	CFPAC-1					79 (32)	18 (−7)	6 (−7)
	DMS-53					67 (−12)	33 (−15)	6 (−15)
AN-246	CFPAC-1					89	28	6
	DMS-53					70	23	2
AN-161	CFPAC-1					95	35	10
	DMS-53					80	39	5
AN-160	CFPAC-1					85	22	0
	DMS-53					68	24	0
AN-251	CFPAC-1					88	26	−2
	DMS-53					60	3	−12
DOX	CFPAC-1					68	22	4
	DMS-53					78	27	1
				Analogs with AN-201				
AN-254	CFPAC-1		95	13	−7			
	DMS-53	45	−6	−15				
AN-247	CFPAC-1		90	27	−7			
	DMS-53	61	4	−14				
AN-257	CFPAC-1		85	16	−7			
	DMS-53	58	0	−18				
AN-215	CFPAC-1		97	42	−7			
	DMS-53	52	2	−15				
AN-252	CFPAC-1		91	56	−6			
	DMS-53	72	13	−19				
AN-201	CFPAC-1		76	8	−5			
	DMS-53	34	−7	−19				

CFPAC-1 cells were incubated in Iscove's modified Dulbecco's medium with 10% fetal bovine serum (FBS) for 120 hr and DMS-53 cells were incubated for 140 hr in Waymouth's MB 752/1 medium containing 10% FBS in 96-well plates. Relative cell number in treated and control plates was determined by crystal violet staining and expressed as T/C values where $T/C = (T - C_0)/(C - C_0) \times 100$. [T = absorbance of treated cultures, C = absorbance of control cultures, C_0 = absorbance of cultures at the start of incubation ($t = 0$). Measured absorbance is proportionate to cell number.] Negative T/C values indicate a cell number smaller than the number originally seeded at $t = 0$—i.e., a cytocidal effect. The structures of the compounds are shown in Table 1. The carrier peptides had no effect on cell proliferation at 10^{-7} M and lower concentrations. T/C values in brackets are derived from results with a sample of AN-253 containing decomposition products.

DOX (unreacted starting material) and its diester that is formed because of the presence of an excess of glutaric anhydride. This improvement in the preparation and purification of *N*-Fmoc-DOX-14-*O*-hemiglutarate resulted in ≈10% higher yields in the conjugation step in comparison with our previous results (35). Cytotoxic BN analogs containing DOX were obtained after cleavage of the Fmoc protecting group. These conjugates were then converted to derivatives with 2-pyrrolino-DOX by reaction with an excess of 4-iodobutyraldehyde (34, 35).

Receptor Binding Affinity. The carrier peptides and their cytotoxic analogs containing DOX and 2-pyrrolino-DOX were tested for their ability to displace the binding of [^{125}I-Tyr^4]BN to BN/GRP receptors on Swiss 3T3 cells. As shown in Table 1, the deletion of the hydrophobic D-amino acids such as D-Phe or D-Tpi from position 6 of carriers B_1 and B_2, respectively, resulted in analogs (B_4 and B_5) that displayed a severe loss of binding affinity. Conjugation of the bulky cytotoxic radicals, containing a very hydrophobic anthracycline moiety, to these shortened carriers led to the formation of analogs with high-binding affinity to BN/GRP receptors on Swiss 3T3 cells. Cytotoxic derivatives of BN antagonist carriers B_2 and B_3, containing D-Phe at position 6, virtually preserved the binding affinity of the carriers, which is in the nanomolar range. However, in the case of carrier B_1 containing D-Tpi at the amino terminus, the attachment of a bulky radical reduced the binding. The binding affinity of AN-254 (2-pyrrolino-DOX-14-*O*-hemiglutarate linked to B_1), was ≈4 times lower than that of the carrier (Table 1).

Cytotoxicity. Antiproliferative activities of the cytotoxic hybrid molecules and their corresponding cytotoxic radicals were compared on CFPAC-1 human pancreatic cancer, DMS-53 human SCLC, PC-3 human prostate cancer, and MKN-45 human gastric cancer cell lines *in vitro* (Tables 2 and 3). The results indicate that the cytotoxic activity of the antineoplastic radicals was virtually preserved in most of the conjugates, the distinct structures showing small variations in their effect on different cell lines. A very high antiproliferative activity of 2-pyrrolino-DOX (AN-201) and its peptide conjugates was observed on DMS-53 cells. As shown in Table 3, AN-201 is ≈2500 times more effective in this cell line than DOX. One of the hybrid analogs, AN-253, consisting of DOX linked to [D-Tpi^6, $^{13}\psi^{14}$,CH$_2$-NH, Leu^{14}]BN-(6-14) (Fig. 1), showed 2–3 times higher antiproliferative activity than DOX when tested after 4 months of storage in a lyophilized form. Thesc data are displaycd in brackets in Table 3. The increased activity was found to be due to decomposition products. Freshly purified AN-253 had a similar activity to DOX. AN-254 consisting of 2-pyrrolino-DOX linked to [D-Tpi^6, $^{13}\psi^{14}$,CH$_2$-NH, Leu^{14}]BN-(6-14) showed a similar instability, but the cytotoxic activity of freshly purified AN-254 did not differ from that of an 80% pure sample. Other hybrid analogs were found to be stable under the same storage conditions.

DISCUSSION

Chemotherapeutic agents play a major role in the management of various cancers in spite of the frequent severe toxic side effects caused by their nonselective action during systemic administration. One of the approaches aimed at improving the selectivity and reducing the toxicity of antitumor agents is drug targeting, which takes advantage of specific receptors for biologically active peptides or macro-

Table 3. Inhibition of growth of CFPAC-1 human pancreatic cancer, DMS-53 human SCLC, PC-3 human prostate cancer, and MKN-45 human gastric cancer cell lines by DOX, 2-pyrrolino-DOX (AN-201), and the corresponding cytotoxic BN analogs

Compound	IC_{50},* 10^{-10} M			
	CFPAC-1 at 120 hr	DMS-53 at 140 hr	PC-3 at 72 hr	MKN-45 at 115 hr
		Analogs with DOX		
AN-253	530 (180)†	640 (≤300)	2100 (760)	2700 (500)
AN-246	650	490	2700	3600†
AN-161	760	780	3500†	5100†
AN-160	580	530	3200†	2300
AN-251	630	370	2600	2000
DOX	570	580	1500	1800
		Analogs with AN-201		
AN-254	1.8	0.33	6.1	2.1
AN-247	2.0	0.37	6.8	2.9
AN-257	1.7	0.35	6.7	2.1
AN-215	2.7	0.41	6.8	2.4
AN-252	3.5	0.46	13.0†	3.7
AN-201	1.6	0.22†	3.6	1.5

*Cell growth inhibition data, determined at three different concentrations as shown in Table 2, were used to calculate the drug concentration that inhibited cell growth by 50%, as compared with untreated control cultures. All data were derived from an average of three determinations each in eight replicates. CFPAC-1 and DMS-53 cells were grown under conditions described in Table 2. PC-3 cells were incubated in RPMI 1640:F12 (1:1) medium containing 1 mM pyruvate/1 μM $FeSO_4$/0.5% bovine serum albumin. MKN-45 cells were incubated in Dulbecco's modified Eagle medium containing 10% FBS.

†Values calculated by extrapolation. IC_{50} values in brackets are derived from results with a sample of AN-253 containing decomposition products.

molecules on the cell membrane of cancerous cells (35, 37). BN-like peptides have properties of hormones or growth factors and are responsible for a wide variety of receptor-mediated pharmacological effects (1). Accordingly, receptors for BN-like peptides are present on normal, nonmalignant cells in the digestive tract, the central nervous system and other target organs such as the lung (1, 2, 10). Investigation of the role of BN-like peptides in the mitogenesis of various cancers revealed that high-affinity binding sites for these peptide hormones are also expressed on a wide variety of human and experimental animal tumors (1–10, 17–32). A recent study also indicates that on certain cancers, such as azaserine-induced pancreatic carcinoma in the rat, high-affinity GRP receptors are present in significantly higher numbers than on the normal pancreas (38). Thus, BN/GRP

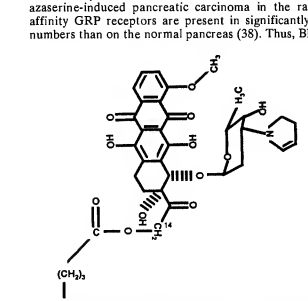

FIG. 1. Molecular structure of cytotoxic BN analog AN-215. 2-Pyrrolino-DOX-14-*O*-hemiglutarate is linked to the N terminal of [$^{13}\Psi^{14}$,CH_2-NH, Leu^{14}]BN-(7-14).

analogs, by virtue of binding to these receptors, may be used for the design of targeted cytotoxic conjugates. The hybrid molecules must preserve both the antineoplastic and specific binding character of their respective components. To create targeted BN-like cytotoxic agents, we linked DOX-14-*O*-hemiglutarate to the N terminal of pseudooctapeptide BN-(7-14) and pseudononapeptide BN-(6-14) analogs previously developed at our institute. The pseudooctapeptide carriers lack the bulky hydrophobic D-amino acids such as D-Phe or D-Tpi at position 6 of BN-(6-14) analogs. As expected, these shortened analogs exhibit no binding to BN/GRP receptors on Swiss 3T3 cells. As shown in Table 1, attachment of a bulky hydrophobic cytotoxic radical to the N terminals of these analogs leads to the formation of cytotoxic BN derivatives with high-binding affinity to BN/GRP receptors. These data show that replacement of the D-amino acids with hydrophobic acids at position 6 of BN-(6-14) analogs can result in BN-(7-14) derivatives with increased binding affinity. Cytotoxic conjugates of pseudononapeptide BN-(6-14) carriers, containing D-Phe at position 6, have high-binding affinity to BN/GRP receptors (Table 1), indicating a tolerance for substitution with bulky groups at the N terminal of these peptides. However, this bulk tolerance of the BN antagonists at the N terminus was not so apparent in the case of carrier B_1 containing D-Tpi at position 6, which is larger than D-Phe.

Antiproliferative activity of the cytotoxic radicals is well preserved in the BN conjugates. Small variations were observed in the cytotoxic activities of different analogs, as compared with the respective cytotoxic radicals incorporated on the four cell lines tested. For instance, DOX showed an activity ≈50% higher on PC-3 prostate cancer cell line than its BN conjugate AN-251, but AN-251 was approximately twice as active on DMS-53 SCLC than DOX (Table 3). Such variations could be due to different binding affinities of the same hybrid analog to receptors on the different cell lines. A sample of AN-253, containing DOX linked to an N-terminal D-Tpi, was ≈2-3 times more potent than DOX on four cancer cell lines when tested after 4 months of storage as a lyophilizate (Tables 2 and 3, values in brackets). A purity

check of this sample by HPLC revealed the presence of several decomposition products. Because a freshly purified sample of AN-253 had similar or even lower antiproliferative effect than DOX, one or more of the decomposition products must be responsible for this increased cytotoxic activity. A plausible explanation of this finding can be given by considering the following. Tpi is formed by Pictet–Spengler condensation of Trp with one equivalent of formaldehyde in dilute acid (39). The less stable *N*-acyl-D-Tpi, present in AN-253, can be expected to undergo decomposition to yield formaldehyde and *N*-acyl-D-Trp. Because vicinal amino alcohols readily react with aldehydes, the amino alcohol function of the daunosamine moiety of DOX would entrap the formaldehyde generated by the *N*-acyl-D-Tpi moiety. Such a byproduct might have a much higher antiproliferative activity than DOX, due to its ability to alkylate a nucleophyl at the intercalation site as clearly demonstrated by Gao *et al* (40). Decomposition products of AN-254 (2-pyrrolino-DOX linked to D-Tpi) could not have increased potency, as compared with the pure product, because 2-pyrrolino-DOX is a latent aldehyde derivative of DOX with increased cytotoxicity. A very high antiproliferative activity of 2-pyrrolino-DOX (AN-201) and its peptide conjugates was observed on DMS-53 SCLC cells. As shown in Table 3, AN-201 is ≈2500 times more active in this cell line than DOX. This great difference in the activity of DOX and its daunosamine-modified derivative is very interesting, because it is not caused by the resistance of DMS-53 to DOX. In fact, of the four cell lines tested, DMS-53 was the most sensitive to DOX (Tables 2 and 3). A high activity of AN-201 suggests that 2-pyrrolino-DOX and its cytotoxic BN conjugates could be used in preference to DOX or its analogs for the treatment of cancers such as SCLC typified by DMS-53.

Preliminary *in vivo* experiments on nitrosamine-induced pancreatic cancers in golden hamsters indicated that both cytotoxic BN analog AN-215 and cytotoxic radical AN-201 have significant antitumor activity in this experimental model. Nevertheless, in one of these pilot experiments, 16 of 18 hamsters died after intraperitoneal administration of a total dose of 100 nmol/kg of cytotoxic radical AN-201 by the 5th week after the last injection. Only 5 of 18 animals died in the group treated with the same dose of AN-215 and 5 of 20 hamsters in the untreated control group. This indicates that the BN hybrid analog AN-215 has lower toxicity than the unconjugated cytotoxic radical.

In conclusion, our *in vitro* studies indicate that BN/GRP analogs linked to DOX or its 2-pyrrolino derivative have high cytotoxic activity. However, additional extensive investigations *in vivo* are required on pancreatic, lung, prostate, gastric, and brain cancer models that possess receptors for BN/GRP, to evaluate the efficacy of these targeted cytotoxic BN analogs.

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Covalently Cyclized Agonist and Antagonist Analogues of Bombesin and Related Peptides*

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During a search for possible cyclization points in shortened, potent bombesin agonists and antagonists, it was found that the joining of amino acid residues in positions 6 and 14 by various means resulted in retention of significant binding affinity for rat pancreatic acini and murine Swiss 3T3 cells. In one series of analogues, Cys residues in these positions were used for bridging via a disulfide bond. (D)-C-Q-W-A-V-G-H-L-C-NH_2 retained significant binding affinity for rat pancreatic acini cells and was a full amylase releasing agonist (EC_{50} 187 nM). Potency was markedly increased by substituting D-Ala for Gly (EC_{50} 67 nM compared to 10 nM for its linear counterpart) and was decreased by substituting L-Cys for D-Cys in this analogue (EC_{50} 214 nM), thus strongly suggesting stabilization of peptide folding by the D residues. Elimination of the COOH-terminal amino acid produces competitive antagonists in the linear analogues; however, (D)-C-Q-W-A-V-G-H-C-NH_2 was devoid of activity. Likewise, cyclization to position 13 with the 14 amino acids intact to give (D)-C-Q-W-A-V-G-H-C-L-NH_2 resulted in an almost inactive peptide. On the other hand, as in the linear series, the reduced peptide bond analogue, (D)-C-Q-W-A-V-(D)-A-H-L-ψ(CH_2NH)-C-NH_2, was a receptor antagonist (IC_{50} 5.7 nM), albeit much weaker than the corresponding linear analogues, but with no residual agonist activity. Direct head-to-tail cyclization was also tried. Both cyclo[(D)-F-Q-W-A-V-G-H-L-L] (EC_{50} 346 nM) and the shorter cyclo[Q-W-A-V-G-H-L-L] (EC_{50} 1236 nM) were full agonists. Elimination of the COOH-terminal residue in cyclo[(D)-p-Cl-F-Q-W-A-V-(D)-A-H-L] produced an agonist (EC_{50} 716 nM) rather than an antagonist. These results provide support for the proposal that both bombesin agonists and antagonists adopt a folded conformation at their receptor(s). Furthermore, the retention of appreciable potencies using several cyclization strategies and chain lengths suggests that further optimization of these structures both in terms of potency and ring size is possible. Since these peptides have increased conformational restriction, they should begin to serve as useful substrates for NMR and molecular modeling studies aimed at comparing the obviously subtle differences between agonist and antagonist structures.

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Competitive receptor antagonists of bombesin (Bn)[1]/GRP peptides have now been created by a number of very different design approaches. Replacement of His in position 12 of Bn (<Q-Q-R-L-G-N-Q-W-A-V-G-H-L-M-NH_2) resulted in the first reported Bn antagonists (1, 2). More potent antagonists were then prepared by replacing the —CONH— peptide bond in position 13–14 by a —CH_2NH— group (3). Moreover, it was discovered that replacement of the 9–10 peptide bond in a similar fashion also resulted in an antagonist, although it exhibited considerably less affinity for guinea pig pancreatic acini cells (3). Elimination (4) of the COOH-terminal Met residue in *N*-Ac-GRP(20–27) (Ac-H-W-A-V-G-H-L-M-NH_2) also gave a receptor antagonist with quite high affinity which was increased substantially by alkylamide, phenalkylamide, and ester modifications at the COOH terminus (4–6). Affinities of both the 13–14 reduced peptide and the desMet analogues were also increased substantially in the Bn series by reducing the chain length to the 6–14 or 6–13 sequences and substituting D-aromatic amino acids in position 6 (7–9). The biological properties of some of these antagonists varied considerably depending on the biological system being employed. Thus, <Q-Q-R-L-G-N-Q-W-A-V-G-H-L-ψ(CH_2NH)-L-NH_2 displayed considerable agonist activity using a frog esophageal peptic cell system (10) and, to a lesser extent, using rat pancreatic acini (8). Even some of the shorter pseudopeptide and peptide desMet[14]-alkylamide analogues were found to have varying degrees of partial or even full agonist activity depending on the structure of the COOH-terminal amino acid or the length of the alkyl substituent (8, 9). In the reduced peptide bond antagonists, COOH-terminal aromatic amino acids had particularly high agonist potency which could, however, be eliminated by halogen substituents on the aromatic side chain (8) or by switching to a D-amino acid. Surprisingly, agonist potency could also be much reduced by a halogen substituent on the D-Phe residue in position 6 (8). Similarly, a 4-Cl-phenylethylamide Bn antagonist displayed little or no agonist activity, whereas the corresponding unsubstituted phenylethylamide had high agonist activity (9).

In more recent studies, it was shown that some of these analogues distinguish Bn receptor subtypes (11). In particular, some reduced peptide bond analogues and various desMet[14] antagonists had much reduced affinity for neuromedin B-preferring Bn receptors on rat esophageal tissue even though their receptors had high affinity for various Bn agonists and neuromedin B itself. Moreover, antagonists based on the D-

[1] The abbreviations used are: Bn, bombesin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hplc, high performance liquid chromatography; ψ, reduced peptide bond; GRP, gastrin-releasing peptide.

Phe[12] modification retained significant affinity for both types of receptor (11).

This complex series of interacting events triggered by diverse structural modifications suggests several conclusions regarding the mechanism of action of these peptides. Clearly, the COOH-terminal dipeptide unit and hydrogen-bonding points integral to this particular peptide bond are critical for triggering the biological response of the peptide agonists and, indeed, we have hypothesized (1) that intramolecular hydrogen bonding could be an important factor in retaining conformational integrity responsible for agonist activity. It seems likely that the apparently diverse structural alterations which result in antagonist formation exercise their effects ultimately through closely related perturbations in this agonist conformation, thus resulting in destruction of biological activity yet with retention of the ability to bind the receptor.

It is extremely difficult to precisely elucidate these conformational parameters by physicochemical measurements on flexible peptide chains which, at least in the case of bombesin and a D-Phe[12] analogue in recent high resolution NMR and CD studies (12, 13), show little ability to adopt preferred conformations in solution other than some evidence for weak α-helicity in the COOH-terminal region of Bn. A partial solution to this problem with peptides is to develop conformationally restricted analogues which are covalently cyclized while retaining as much affinity for their receptors as possible. Establishing those initial structural leads which enable potency to be retained after cyclization is often difficult; however, early attempts to design cyclic Bn analogues did result in a moderately active agonist when positions 5–14 were joined (14) in a 4–14 structure, thus suggesting the feasibility of this type of approach. In the present study, we were also intrigued by the observations described above in which modifications to NH_2- and COOH-terminal aromatic amino acid residues in Bn(6–14) analogues resulted in similar effects on biological activity. We reasoned that their side chains could be in close proximity while bound to receptors and that possibly cyclization from position 6–14 might be of some interest. A number of cyclization strategies are described utilizing short agonist and antagonist structures as starting points.

EXPERIMENTAL PROCEDURES

Materials

Protected amino acids were obtained from Bachem, Inc., Torrance, CA. Rats were obtained from the Small Animals Section, Veterinary Resources Branch, NIH. HEPES was from Boehringer Mannheim; purified collagenase (type CLSPA, 440 units/mg) from Worthington Biochemicals; sodium borate, soybean trypsin inhibitor, carbamylcholine, theophylline, bacitracin from Sigma; essential vitamin mixture (100× concentrated) from Microbiological Associates, Bethesda, MD; Na[125]I from Amersham Corp.; Phadebas amylase test reagent from Pharmacia LKB Biotechnology Inc.; bovine plasma albumin (Fraction V) from Miles Laborstories; COOH-terminal octapeptide of cholecystokinin (CCK-8) from Peninsula Laboratories, Belmont, CA; and FURA-2/AM from Molecular Probes, Eugene, OR. Stock cultures of murine Swiss 3T3 cells were kindly provided by Dr. E. Rozengurt, Imperial Cancer Research Fund, London.

The standard incubation solution used in experiments involving pancreatic acini contained 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM theophylline, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) amino acid mixture, and 1% (v/v) essential vitamin mixture. The incubation solution was equilibrated with 100% O_2, and all incubations were carried out with O_2 as the gas phase.

Methods

Preparation of Peptides—Automated solid phase syntheses of peptides (Advanced ChemTech Model 200), including introduction of the reduced peptide bond, were carried out by the standard methods recently described by us (3) on methylbenzhydrylamine resin (Advanced ChemTech, Louisville, KY) for COOH-terminal amides and standard Leu-*O*-polystyrene resin for analogues with free carboxyl-terminal COOH groups prior to cyclization. The single analogue containing the reduced peptide bond was synthesized by the method of Sasaki and Coy (15). The crude hydrogen fluoride-cleaved Cys peptide amides were cyclized in 90% acetic acid solution by titration with dilute I_2 solution prior to purification. These materials and peptide-free acids, prior to NH_2- to COOH-terminal cyclization, were purified on a column (2.5 × 90 cm) of Sephadex G-25 which was eluted with 2 M acetic acid followed by preparative medium pressure chromatography on a column (1.5 × 45 cm) of Vydac C18 silica (10–15 μm) which was eluted with linear gradients of acetonitrile in 0.1% trifluoroacetic acid using a Rainin (Woburn, MA) hplc system with a Macintosh computer controller (flow rate approximately 2 ml/min). Analogues were further purified by re-chromatography on the same column with slight modifications to the gradient conditions when necessary. Homogeneity of the peptides was assessed by thin layer chromatography and analytical reverse-phase high pressure liquid chromatography, and purity was 97% or higher. Peptides with free termini were then cyclized in dilute dimethylformamide solution by treatment with a small excess of dicyclohexylcarbodiimide/1-hydroxybenzotriazole or BOP reagent/diisopropylethylamine and monitoring by analytical hplc. They were further purified as already described.

Amino acid analysis of acid hydrolysates of the peptides gave the expected amino acid ratios. The primary structures of the cyclic peptides were further confirmed by fast atom bombardment mass spectrometry, and each gave the expected molecular ion.

Tissue Preparation—Dispersed acini from rat pancreas were prepared as described previously (9).

Amylase Release—Dispersed acini from one rat pancreas were suspended in 150 ml of standard incubation solution, samples (250 ml) were incubated for 30 min at 37 °C, and amylase release was measured as described previously (16, 17). Amylase activity was determined by the method of Ceska *et al.* (18, 19) using the Phadebas reagent. Amylase release was calculated as the percentage of amylase activity in the acini at the beginning of the incubation that was released into the extracellular medium during the incubation.

Effect of Peptides on Bombesin-stimulated Amylase Release—Antagonist activity was determined as described before (9). Various concentrations of peptides were incubated alone or with 0.3 nM bombesin, a concentration that causes half-maximal stimulation which, in the present studies, was a 5.5-fold increase over basal.

Binding of [125]I-Labeled [Tyr[4]]Bombesin to Acini or 3T3 Fibroblasts—[125]I-[Tyr[4]]bombesin (2000 Ci/mmol) was prepared by the method described previously (20). [125]I-[Tyr[4]]bombesin was separated from [125]I using a Sep-Pak and separated from unlabeled peptide by reverse phase high pressure liquid chromatography on a column (0.46 × 25 cm) of μBondapak C18. The column was eluted isocratically with acetonitrile (22.5%) and triethylammonium phosphate (0.25 M (pH 3.5)) (77.5%) at a flow rate of 1 ml/min. Incubations contained 0.05 nM [125]I-[Tyr[4]]bombesin and were for 60 min at 37 °C for pancreatic acini and for 30 min at 22° C for 3T3 cells.

Nonsaturable binding of [125]I-[Tyr[4]]bombesin was the amount of radioactivity associated with the acini or 3T3 cells when incubation contained 0.05 nM [125]I-[Tyr[4]]bombesin plus 1 mM bombesin. All values shown are for saturable binding, *i.e.* binding measured with [125]I-[Tyr[4]]bombesin alone (total) minus binding measured in the presence of 1 mM unlabeled bombesin (nonsaturable binding). Nonsaturable binding was <10% of total binding in all experiments.

Effects of Peptides on Cytosolic Calcium in Swiss 3T3 Fibroblasts—Swiss 3T3 cells (3×10^{-6} cells/ml) were loaded with 2 mM FURA-2/AM for 45 min at 37° C in standard incubation buffer without phosphate or essential vitamin mixture. Cytosolic Ca^{2+}, $[Ca^{2+}]_i$, was measured using a Delta PTI Scan-1 spectrofluorimeter (PTI Instruments, Gaithersburg, MD) which had provisions for continuous stirring and temperature control at 37 °C. Fluorescence was measured at 500 nm after excitation at 340 nm and 380 nm. Autofluorescence of the unloaded cells was subtracted, and $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz *et al.* (21).

RESULTS

When tested at a concentration of 10 nM, 9 of the 12 cyclized Bn analogues stimulated amylase release from the rat acinar cells (Table I). Three analogues, [$Cys^{9,14}$]Bn, [D-Cys^6,Cys^{13}]Bn(6–13), and [D-Cys^6,D-Ala^{11},Cys^{14},ψ^{13-14}]Bn(6–14) failed to stimulate amylase release (Table I). When each of these peptides was studied for antagonist properties by determining its ability to inhibit Bn-stimulated amylase release, only the latter caused inhibition, whereas the other two were devoid of effect (Table I, right column).

Dose-response curves illustrating the relative abilities of the cyclic analogues and several control peptides to stimulate or inhibit Bn-stimulated amylase release from these cells are shown in Fig. 1 and Fig. 2, respectively, and their ability to inhibit ^{125}I-[Tyr^4]Bn binding to Bn receptors on rat pancreatic acini cells is shown in Fig. 3. The EC_{50} and K_i values calculated from these data are given in Table II. Since all but one of the cyclic structures were based on either the Bn(6–14) sequence (N-Q-W-A-V-G-H-L-M-NH_2), the 6–14 sequence containing the reduced peptide bond between positions 13 and 14, on litorin (<Q-Q-W-A-V-G-H-F-M-NH_2), which is closely related to Bn(6–14), containing the ψ-bond between position 8 and 9, or the $desMet^{14}$-(6–13) sequence of Bn, a number of linear control peptides were included to encompass these sequences. These included Bn itself (I, Table II), [Leu^{13}]Bn (II), Bn(6–14) (III), [D-Phe^6]Bn(6–14) (IV), [D-Phe^6,D-Ala^{11},Leu^{14}]Bn (VII), litorin (IX), and [$Leu^{8,9}$]litorin (X), all of which were potent agonists with high affinities and K_i values ranging from 2–78 nM (Table II). Control representatives of previously described linear antagonists included [D-Phe^6,Leu^{14},ψ^{13-14}]Bn(6–14) (XXIII) (IC_{50} 40 nM), Bn(6–13) (XVIII) (IC_{50} 5.2 mM), [D-Phe^6]Bn(6–13) (XIX) (IC_{50} 0.1 mM), and [Leu^8,$desMet^9$]litorin (XXI) (IC_{50} 0.77 mM).

All cyclic and control linear peptides, except two cyclic peptides, [D-Cys^6,Cys^{13}]Bn(6–13) (XX) and [$Cys^{9,14}$]Bn (XVII) and the linear peptide Bn(9–14) (XV), interacted with Bn receptors to either stimulate or inhibit Bn-stimulated amylase release (Figs. 1–3). In contrast to [D-Cys^6,Cys^{13}]-Bn(6–13) (XX, Table II), the linear peptides [D-Phe^6]Bn(6–13) (XIX) or Bn(6–13) (XVIII) both functioned as receptor antagonists (Fig. 2 and Table II). Agonist activity and binding affinity were restored to XX by the addition of a Leu substituent to position 14 to give [D- Cys^6,Cys^{13},Leu^{14}]Bn(6–14) (XIII) which still had very low affinity and biological potency (EC_{50} 5.5 mM; K_i = 28 mM) compared to the linear peptide [D-Phe^6]Bn(6–14) (EC_{50} 0.0006 mM; Ki = 0.006 mM) (Figs. 1 and 3; Table II). Cyclization between positions 6 and 14 via a disulfide bridge in [D-Cys^6,Cys^{14}]Bn(6–14) (VIII) gave a much more potent agonist analogue (EC_{50} 0.19 mM; K_i = 3.3 mM), and agonist potency was increased still further by the presence of D-Ala in place of Gly^{11} to give [D-Cys^6,D-Ala^{11},Cys^{14}]Bn(6–14) (VI) (EC_{50} 67 nM; K_i = 69 nM) (Figs. 1 and 3; Table II). Potency was partially lost if L-Cys was employed rather than D-Cys in position 6 and [Cys^6,D-Ala^{11},Cys^{14}]Bn(6–14) (V) had an EC_{50} of 0.21 mM and a K_i of 2.1 mM. Cyclization between

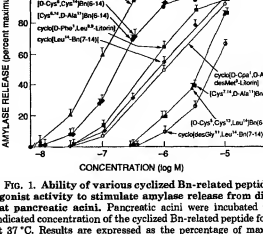

FIG. 1. **Ability of various cyclized Bn-related peptides with agonist activity to stimulate amylase release from dispersed rat pancreatic acini.** Pancreatic acini were incubated with the indicated concentration of the cyclized Bn-related peptide for 30 min at 37 °C. Results are expressed as the percentage of maximal responses by 10 nM Bn. Basal and maximal amylase release was 2.5 ± 0.1% and 19.6 ± 1.8% of total cellular amylase release, respectively. Each *point* is a mean from four separate experiments, and, in each experiment, each value was determined in duplicate. *Vertical bars* represent 1 S.E.

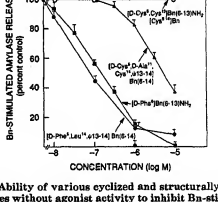

FIG. 2. **Ability of various cyclized and structurally related Bn peptides without agonist activity to inhibit Bn-stimulated amylase release.** Pancreatic acini were incubated with or without 0.3 nM Bn, a half-maximally effective concentration, with the indicated concentrations of the various Bn-related peptides. Results are expressed as the percentage of the stimulated amylase release caused by 0.3 nM alone. Basal and 0.3 nM Bn-stimulated amylase release was 2.6 ± 0.1% and 14.6 ± 2.0% of the total cellular amylase release, respectively. Each *point* is a mean from four separate experiments, and, in each experiment, each value was determined in duplicate. *Vertical bars* represent 1 S.E.

TABLE I

Effect of various cyclized Bn analogues on basal and Bn-stimulated amylase release from rat pancreatic acini cells

Pancreatic acini were incubated at 37 °C for 30 min either alone, with 0.3 nM Bn, or a 10 μM concentration of the indicated concentration of the cyclized Bn analogue alone or in combination. Values are means ± 1 S.E. from five separate experiments. Values for amylase release are expressed as the percentage of the total cellular amylase that was released into the extracellular medium during the incubation. NT-agonist = cyclized analogue was not tested for antagonist activity because it had agonist activity.

Peptide added (10 μM)	Amylase release	
	Alone	Plus 0.3 nM Bn
	% total	
None	2.5 ± 0.1	14.6 ± 2.1
[$Cys^{6,14}$,D-Ala^{11}]Bn(6–14)	19.1 ± 0.6[a]	NT-agonist
[D-Cys^6,D-Ala^{11},Lys^{14}]Bn(6–14)	18.1 ± 1.0[a]	NT-agonist
[D-Cys^6,Cys^{14}]Bn(6–14)	19.9 ± 1.1[a]	NT-agonist
Cyclo[D-Phe^6,$Leu^{6,9}$]Litorin	17.9 ± 1.4[a]	NT-agonist
[Cys^{7-14},D-Ala^{11}]Bn(6–14)	17.0 ± 1.9[a]	NT-agonist
[D-Cys^6,Cys^{14},Leu^{14}]Bn(6–14)	16.9 ± 1.2[a]	NT-agonist
Cyclo[Leu^{14}-Bn(7–14)]	20.5 ± 2.5[a]	NT-agonist
Cyclo[desGly^{11},Leu^{14}]Bn(7–14)	13.6 ± 0.2[a]	NT-agonist
[$Cys^{9,14}$]Bn	2.7 ± 0.1[a]	13.9 ± 1.1
[D-Cys^6,Cys^{13}]Bn(6–13)	2.6 ± 0.2	14.2 ± 0.7
Cyclo[Cys^6,D-Ala^{11},Leu^{14},$desMet^{14}$]litorin	17.5 ± 1.4[a]	NT-agonist
[D-Cys^6,D-Ala^{11},Cys^{14},ψ^{13-14}]Bn(6–14)	2.6 ± 0.2	6.8 ± 1.1[a]

[a] Significantly greater than no additions, $p < 0.01$.

[b] Significantly less than 0.3 nM Bn alone, $p < 0.01$.

positions 7 and 14 to give [Cys^7,D-Ala^{11},Cys^{14}]Bn(6–14) (XII) resulted in a relatively weakly active agonist (EC_{50} 2.1 mM; K_i = 45 mM) (Figs. 1 and 3; Table II). Cyclization of a D-Phe^1 version of [$Leu^{8,9}$]litorin via the α-amino group and the COOH-terminal COOH group to give cyclo[D-Phe^1,$Leu^{8,9}$] litorin (XI) retained almost all of the agonist potency (EC_{50} 0.35 mM; K_i = 1.2 mM) of the former (Table II). The related shorter 7–14 analogue, cyclo[Leu^{14}]Bn(7–14) (XIV) was only a 3 times less potent agonist, and even removal of Gly^{11} in this to give cyclo[desGly^{11},Leu^{14}]Bn(7–14) (XV) resulted in retention of some agonist activity (EC_{50} 6.5 mM; K_i = 25.8 mM) (Figs. 1 and 3; Table II). Elimination of the COOH-terminal Met residue to give cyclo[D-Cpa^1,$Leu^{8,9}$desMet^9]litorin (XXII) resulted in the most potent agonist of this series (EC_{50} 0.7 mM; K_i = 0.4 mM) (Figs. 1 and 3; Table II).

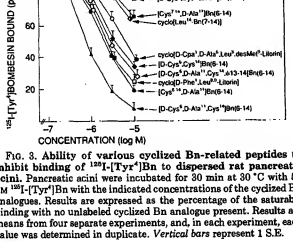

FIG. 3. **Ability of various cyclized Bn-related peptides to inhibit binding of ^{125}I-[Tyr^4]Bn to dispersed rat pancreatic acini.** Pancreatic acini were incubated for 30 min at 30 °C with 50 pM ^{125}I-[Tyr^4]Bn with the indicated concentrations of the cyclized Bn analogues. Results are expressed as the percentage of the saturable binding with no unlabeled cyclized Bn analogue present. Results are means from four separate experiments, and, in each experiment, each value was determined in duplicate. *Vertical bars* represent 1 S.E.

To confirm that the various cyclized Bn analogues that functioned as agonists were actually stimulating amylase release through Bn receptor occupation, the effect of the specific Bn receptor antagonist, [D-Phe^6]Bn(6–13) methyl ester, on stimulation by the most potent cyclic agonist, [D-Cys^6,D-Ala^{11},Cys^{14}]Bn(6–14) was examined (Fig. 4). At a 2 nM dose level, the methyl ester caused half-maximal inhibition of amylase release produced by a 0.1 mM concentration of the cyclic agonist, whereas a 0.1 mM dose produced complete blockade of amylase release. Increasing the cyclic agonist concentration to 1 mM resulted in a typical parallel shift in the dose-response inhibition curve, indicating that the agonist/antagonist were interacting in a competitive manner.

Replacement of the position 13–14 peptide bond in the most potent agonist analogue of the Cys-bridged series (VI) by a CH_2NH group to give [D-Cys^6,D-Ala^{11},Cys^{14},ψ^{13-14}]Bn(6–14) (XXIV) resulted in destruction of agonist activity; however, the peptide still retained binding affinity and exhibited a K_i of 2.2 mM (Figs. 1–3; Table II). This peptide was able to inhibit the amylase-releasing response of Bn itself (Fig. 2) with an IC_{50} of 5.7 mM. The inhibitory effect of this cyclic antagonist on Bn-stimulated amylase release was investigated in more detail (Fig. 5) using acini incubated with various doses of Bn. Detectable amylase release occurred with 0.03 nM Bn, half-maximal at 0.3 nM, and maximal at 10 nM. [D-Cys^6,D-Ala^{11},Cys^{14},ψ^{13-14}]Bn(6–14) caused a parallel rightward shift in the dose-response curve for Bn-stimulated amylase release, and this was proportional to the concentration of the analogue used (Fig. 5). There was no change in the maximal Bn response (Fig. 5). Analysis of these data by the Schild

TABLE II

Bioactivities and affinities of cyclized and other structurally related Bn analogues for Bn receptors on rat pancreatic acini cells

K_i values for binding of the various analogues were calculated by the method of Cheng and Prusoff (28) and are from the data shown in Fig. 3. The EC_{50} or IC_{50} values are from the data shown in Figs. 1 and 2 and represent the concentration causing half-maximal amylase release or half-maximal inhibition of Bn-stimulated amylase release, respectively. Each value is the mean ± 1 S.E. of at least four experiments. Abbreviations: Antag = antagonist, Ag = agonist, P. Ag (% max) = partial agonist (percent maximal response), Cpa = *p*-chlorophenylalanine.

	Peptide	Biological activity	EC_{50}/IC_{50}	K_i
		μM	μM	
I	Bn	Ag	0.0002 ± 0.0001	0.004 ± 0.001
II	[Leu^{14}]Bn	Ag	0.0008 ± 0.0002	0.021 ± 0.004
III	Bn(6–14)	Ag	0.0002 ± 0.0001	0.006 ± 0.001
IV	[D-Phe^6]Bn(6–14)	Ag	0.0006 ± 0.0001	0.002 ± 0.001
V	[$Cys^{6,14}$,D-Ala^{11}]Bn(6–14)	Ag	0.21 ± 0.03	2.1 ± 0.3
VI	[D-Cys^6,D-Ala^{11},Cys^{14}]Bn(6–14)	Ag	0.067 ± 0.006	0.69 ± 0.09
VII	[D-Phe^6,D-Ala^{11},Leu^{14}]Bn(6–14)	Ag	0.010 ± 0.002	0.013 ± 0.003
VIII	[D-Cys^6,Cys^{14}]Bn(6–14)	Ag	0.19 ± 0.01	3.3 ± 0.7
IX	Litorin	Ag	0.0004 ± 0.0001	0.006 ± 0.001
X	[$Leu^{8,9}$]Litorin	Ag	0.49 ± 0.13	0.78 ± 0.41
XI	Cyclo[D-Phe^1,$Leu^{8,9}$]litorin	Ag	0.35 ± 0.04	1.2 ± 0.1
XII	[Cys^{7-14},D-Ala^{11}]Bn(6–14)	Ag	2.1 ± 0.1	45.2 ± 10.6
XIII	[D-Cys^6,Cys^{13},Leu^{14}]Bn(6–14)	Ag	5.5 ± 0.8	28.2 ± 10.2
XIV	Cyclo[Leu^{14}-Bn(7–14)]	Ag	1.2 ± 0.4	4.0 ± 0.8
XV	Cyclo[des-Gly^{11},Leu^{14}-Bn(7–14)]	Ag	6.5 ± 0.6	25.8 ± 12.2
XVI	Bn(9–14)	None	No activity at 10	>10
XVII	[Cys^{9-14}]Bn	None	No activity at 10	>30
XVIII	Bn(6–13)	Antag	5.2 ± 1.4	9.2 ± 2.5
XIX	[D-Phe^6]Bn(6–13)	Antag	0.10 ± 0.02	0.03 ± 0.01
XX	[D-Cys^6,Cys^{13}]Bn(6–13)	None	No activity at 10	58.4 ± 20.6
XXI	[Leu^9,desMet^9]litorin	Antag	0.7 ± 0.12	0.32 ± 0.15
XXII	Cyclo[Cpa^1,D-Ala^8,Leu^9,desMet^9]litorin	Ag	0.7 ± 0.2	0.4 ± 1.8
XXIII	[D-Phe^6,Leu^{14},ψ^{13-14}]Bn(6–14)	P. Ag (10% max)	0.040 ± 0.012	0.062 ± 0.001
XXIV	[D-Cys^6,D-Ala^{11},Cys^{14},ψ^{13-14}]Bn(6–14)	Antag	5.7 ± 2.7	2.2 ± 0.3

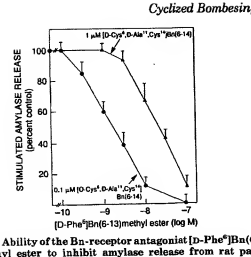

FIG. 4. Ability of the Bn-receptor antagonist [D-Phe⁶]Bn(6–13) methyl ester to inhibit amylase release from rat pancreatic acini stimulated by [D-Cys⁶,D-Ala¹¹, Cys¹⁴]Bn(6–14). Pancreatic acini were incubated with or without either 0.1 μM or 1 μM [D-Cys⁶,D-Ala¹¹,Cys¹⁴]Bn(6–14) with the indicated concentrations of [D-Phe⁶]Bn(6–13) methyl ester for 30 min at 37 °C. Values are expressed as the percentage of control which is the stimulated amylase release caused by 0.1 μM or 1 μM [D-Cys⁶,D-Ala¹¹,Cys¹⁴]Bn(6–14) alone. Basal, 0.1 μM, or 1 μM [D-Cys⁶,D-Ala¹¹,Cys¹⁴]Bn(6–14) caused 3.6 ± 0.6, 14.6 ± 6.7, and 18.7 ± 2.4% of total cellular amylase. Each value is the mean of three separate experiments, and, in each experiment, each point is determined in duplicate. *Vertical bars* represent 1 S.E.

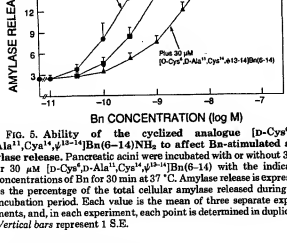

FIG. 5. Ability of the cyclized analogue [D-Cys⁶,D-Ala¹¹,Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14)NH_2 to affect Bn-stimulated amylase release. Pancreatic acini were incubated with or without 3 μM or 30 μM [D-Cys⁶,D-Ala¹¹,Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14) with the indicated concentrations of Bn for 30 min at 37 °C. Amylase release is expressed as the percentage of the total cellular amylase released during the incubation period. Each value is the mean of three separate experiments, and, in each experiment, each point is determined in duplicate. *Vertical bars* represent 1 S.E.

method gave the regression equation, $y = 1.07(\pm 0.05)x + 6.175$ with a correlation coefficient of 0.99 ($p < 0.001$) which has a slope not significantly different from unity and gave a binding affinity of 1.7 ± 0.1 nM. The specificity of the inhibitory activity of [D-Cys⁶,D-Ala¹¹,Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14) to inhibit the action of a number of agonists that do not interact with Bn receptors was studied (Table III). The analogue did not inhibit amylase release produced by CCK-8, carbachol, or secretin at 10 nM, a concentration which significantly inhibited Bn or neuromedin B-stimulated secretion (Table III).

In previous studies (22), Bn-related peptides have been shown to interact with high affinity receptors on 3T3 cells

TABLE III

Effect of [D-Cys⁶,D-Ala¹¹,Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14) on the ability of various agents to stimulate enzyme secretion from rat pancreatic acini

Pancreatic acini were incubated for 30 min at 37 °C with the indicated secretagogue either alone or with 10 μM [D-Cys⁶,D-Ala¹¹,Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14). Results are expressed as the percentage of the total cellular amylase released into the extracellular medium during the incubation. Results are means ± 1 S.E. from five experiments, and in each experiment each value was determined in duplicate.

Secretagogue	Amylase release	
	Alone	Plus 10 μM [D-Cys⁶,D-Ala¹¹, Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14)
	% *total*	
None	4.0 ± 0.5	4.4 ± 0.8
Bn (0.3 nM)	15.0 ± 1.0	7.4 ± 2.0*
Neuromedin B (30 nM)	16.4 ± 2.0	5.7 ± 1.2*
CCK-8 (0.1 nM)	19.3 ± 2.4	18.6 ± 1.9
Carbachol (0.3 μM)	15.7 ± 2.0	16.7 ± 1.7
Secretin (0.1 μM)	9.1 ± 1.0	12.1 ± 2.0

* Significantly different from secretagogue alone, $p < 0.01$.

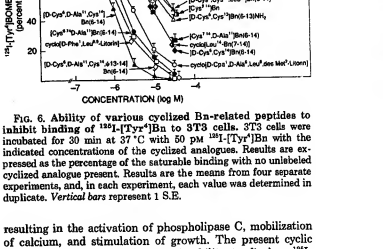

FIG. 6. Ability of various cyclized Bn-related peptides to inhibit binding of ^{125}I-[Tyr⁴]Bn to 3T3 cells. 3T3 cells were incubated for 30 min at 37 °C with 50 pM ^{125}I-[Tyr⁴]Bn with the indicated concentrations of the cyclized analogues. Results are expressed as the percentage of the saturable binding with no unlabeled cyclized analogue present. Results are the means from four separate experiments, and, in each experiment, each value was determined in duplicate. *Vertical bars* represent 1 S.E.

resulting in the activation of phospholipase C, mobilization of calcium, and stimulation of growth. The present cyclic analogues were examined for their ability to displace ^{125}I-[Tyr⁴]Bn from intact 3T3 cells (Fig. 6) or alter cytosolic Ca^{2+} in 3T3 cells (Fig. 7). In a similar fashion to Bn receptors on pancreatic acini, [D-Cys⁶,D-Ala¹¹,Cys¹⁴]Bn(6–14) (K_i = 0.95 nM) had the highest affinity of all the cyclic analogues. Furthermore, each of the analogues had the same relative potencies for Bn receptors on both cell types (compare Figs. 3 and 6). [D-Cys⁶,D-Ala¹¹,Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14) also bound to Bn receptors on 3T3 cells with a 2-fold higher affinity than on rat acinar cells (K_i = 1.0 ± 0.1 *versus* 2.2 ± 0.3 nM, respectively). To investigate whether these analogues were functionally active on 3T3 cells, the abilities of [D-Cys⁶,D-Ala¹¹,Cys¹⁴]Bn(6–14), which functions as an agonist in pancreatic acini, and [D-Cys⁶,D-Ala¹¹,Cys¹⁴,ψ¹³⁻¹⁴]Bn(6–14), which functions as an antagonist in acini, to alter cytosolic Ca^{2+} in 3T3 cells were determined (Fig. 7). The former caused a dose-dependent increase in $[Ca^{2+}]_i$ with a half-maximal effect at 1 nM (Fig. 7, *right*), whereas the latter peptide had no effect at doses up to 30 nM (Fig. 7, *middle*) but caused a dose-depend-

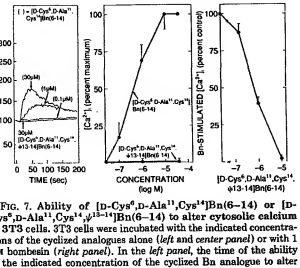

FIG. 7. Ability of [D-Cys^8,D-Ala^{11},Cys^{14}]Bn(6–14) or [D-Cys^8,D-Ala^{11},Cys^{14},ψ^{13-14}]Bn(6–14) to alter cytosolic calcium in 3T3 cells. 3T3 cells were incubated with the indicated concentrations of the cyclized analogues alone (*left* and *center panel*) or with 1 nM bombesin (*right panel*). In the *left panel*, the time of the ability of the indicated concentration of the cyclized Bn analogue to alter cytosolic calcium is shown, and, in the *middle panel*, the results are expressed as the percentage of the maximal response by 30 μM [D-Cys^8,D-Ala^{11},Cys^{14}]Bn(6–14). In these experiments, the control value of cytosolic calcium was 98 ± 9, and the 30 μM [D-Cys^8,D-Ala^{11}, Cys^{14}]Bn(6–14) response was 221 ± 4. In the *right panel* is shown the ability of various concentrations of [D-Cys^8,D-Ala^{11},Cys^{14},ψ^{13-14}]Bn(6–14) to inhibit the increase in cytosolic calcium caused by 1 nM bombesin. In these experiments, basal calcium concentration was 113 ± 5, and 1 nM stimulated the cytosolic calcium to 233 ± 5 (n = 5). *Vertical bars* represent 1 S.E.

ent inhibition of the Bn-stimulated increase in [Ca^{2+}]$_i$ with a half-maximal effect at 1 nM (Fig. 7, *right*).

DISCUSSION

Cyclization of normally flexible, linear peptides has become a standard approach in analogue design and can result in interesting effects on biological properties, *in vivo* stability, and specificity and also yield much useful information on the mechanism of action of the ligand which, even in solution, would necessarily have a closer conformational resemblance to its receptor-bound state as long as reasonable affinity was maintained. The technique has been tried on numerous peptide systems and, in particular, work on cyclic enkephalin analogues with disulfide bridges (23), δ receptor specificity, and luteinizing hormone-releasing hormone analogues with multiple amide bridges (24) has resulted in analogues with sufficient rigidity to be useful for NMR conformational studies.

In the present Bn study, the already discussed choice of positions 6 and 14 as cyclization points appears to be sound in that it has resulted in retention of significant binding affinity with (D)-C-Q-W-A-V-G-H-L-C-NH$_2$ (VIII), although this was roughly 1000 times less than its linear counterpart, [D-Phe^6]Bn(6–14) (IV) for the rat acini cells. The ring size significantly affected affinity because the 7–14 cyclic, N-C-W-A-V-(D)-A-H-L-C-NH2 (XII), underwent a considerably greater loss of affinity, and the 9–14 analogue (XVII) had no activity. The fact that the affinity of VIII could be increased 5-fold by substitution of D-Ala for Gly in position 11 to give (D)-C-Q-W-A-V-(D)-A-H-L-C-NH$_2$ (VI) is strongly indicative of stabilization of folding by, perhaps, β-turn formation. This position in the linear peptides has previously been proposed (1, 25) as a potential folding site which might be stabilized by D-Ala; however, in the linear peptides, little effect on potency is generally observed. In the present series of peptides, [D-Phe^6,D-Ala^{11},Leu^{14}]Bn (VII) is actually one-half as potent as [D-Phe^6]Bn(6–14) (IV) (Table II), and this can probably be attributed to the Leu^{14} substitution (also compare I and II). The importance of a D-amino acid in position 6 also is illustrated by the over 3-fold loss of potency and binding affinity with C-Q-A-V-(D)-A-L-C-NH$_2$ (V), thus suggesting the possibility of folding in this region of the peptide backbone as well. High resolution NMR studies are being attempted on the Cys-bridged agonist and antagonist analogues (VI and XXIV, respectively) using peptide solutions in MeOH, dimethyl sulfoxide, and dimethyl sulfoxide/H_2O at various temperatures.[2] The quality of the one-dimensional spectra has generally been poor due to line broadening at all temperatures, making complete structural assignments difficult. The two-dimensional spectra yielded better data, and cyclization between the 2 Cys residues could be shown by NOEs between [Cys^{14}C$^{\alpha}$H]-[D-Cys^{6}C$^{\alpha}$H] and [Cys^{14}NH$_2$]-[D-Cys^{6}C$^{\alpha}$H]. However, too few nonsequential NOEs have been observed for the presence of specific turns or conformations to be proposed. It should be emphasized, however, that even when cyclized these quite large sequences still possess relatively little conformational restraint and thus retain much of the flexibility of the linear peptides in solution. Meaningful NMR measurements aimed at elucidating possible receptor-bound conformations thus requires the introduction of much additional structural simplification and restraint for which the present analogues provide a useful basis.

In the present synthetic studies, the disulfide bridge strategy involving linked amino acid side chains was not the only successful method for producing biologically active cyclic analogues. The joining of termini in the Bn(6–14) series or 1–8 litorin series via a normal peptide bond also retained affinity and amylase releasing activity. Cyclo[(D)-F-Q-W-A-V-G-H-L-L] (XI, Table II) was about as potent as its Cys-bridged counterpart (VIII). Shortening the chain to 8 amino acids by removal of 1 NH$_2$-terminal residue to give cyclo[Q-W-A-V-G-H-L-L] (XIV) resulted in little loss of activity, and even further shortening by removal of the Gly residue to give cyclo[Q-W-A-V-H-L-L] (XV) resulted in the surprising retention of significant potency.

We were also interested in designing constricted Bn receptor antagonists by approaches similar to those used previously (26) in the linear analogues, that is by either removal of the NH$_2$-terminal amino acid or incorporation of the CH$_2$NH bond between the 2 COOH-terminal amino acids. Elimination of the COOH-terminal amino acid in the Cys-bridged series with [D-Tyr^8,Cys^{13}]Bn(6–13) (XIX) resulted in almost complete loss of affinity for both rat pancreatic acini and 3T3 cells and probably reflects the incompatibility of the position 13 side chain to derivatization in this manner since readdition of the position 14 amino acid in XIII resulted in only a partial regaining of affinity. Elimination of the COOH-terminal amino acid in the head-to-tail cyclics to give cyclo[(D)-*p*-Cl-F-Q-W-A-V-G-H-L] (XXII) resulted in complete retention of binding affinity and agonist potency. In this structure, however, the position 6 amino acid becomes synonymous with the position 14 residue so that it can be considered to be simply a NH$_2$-terminally shortened analogue containing a D- amino acid in position 14.

The reduced peptide bond approach using the most potent Cys-bridged cyclic agonist analogue (VI) as the base structure was much more successful in producing an antagonist. Incorporation of a reduced peptide bond between positions 13 and 14 converted it from an agonist to full antagonist (XXIV) with only about a 3-fold loss of binding affinity. A number of observations supported the conclusion that this cyclic peptide was inhibiting the action of Bn by functioning as a Bn-

[2] G. Van Binst and P. Verheyden, personal communication.

receptor antagonist. The inhibitory activity was only observed for the Bn-receptor agonists, Bn and neuromedin B, and not for other pancreatic secretagogues such as CCK-8 and secretin which stimulate secretion through different receptors. A Schild plot of the ability of the cyclic antagonist to inhibit the action of Bn on pancreatic acini gave a slope not significantly different from unity, thus suggesting it was behaving as a classical Bn receptor antagonist. Also, for both 3T3 cells and rat pancreatic acini, the dose-inhibition curves for altering Bn-induced changes in biological activity and inhibiting ^{125}I-labeled Bn extended over the same concentration ranges which demonstrates directly that inhibitory activity is attributable to Bn receptor occupation. Interestingly, this antagonist was devoid of detectable partial agonist activity, whereas its linear counterpart, [D-Phe^6,Leu$^{13}\psi$(CH$_2$NH)Leu^{14}]Bn(6–14) (XXIII) has been shown to retain partial agonist activity (8).

We have theorized previously (6) that the conversion of an agonist to an antagonist structure by replacement of the 13–14 peptide bond was in some way related to the loss of the normal peptide bond CO group and a concomitant hydrogen bond with a peptide bond NH group present in another part of the peptide chain. This could result in greater rotational freedom at the COOH terminus and destruction of the correct agonist conformation via side chain shifts. It could be argued that antagonist formation by insertion of the pseudopeptide bond in a cyclic peptide in which free rotation is already suppressed by the disulfide bridge does not support this conclusion. However, as already mentioned, these large cyclic peptides still possess much flexibility so that loss of a transannular hydrogen bond could still produce sufficient conformational change for bioactivity to be lost.

In summary, a number of biologically active cyclic bombesin receptor agonist and antagonist analogues have been synthesized and characterized which comprise from 7–9 amino acid residues. The success of a variety of cyclization strategies, chain sizes, and additional conformational restraint via the use of D-amino acid suggests that further structural simplification and potency increases can be obtained. There is much literature precedent for this; for instance, the cyclic somatostatin tetradecapeptide was finally reduced to a 6-residue cyclic structure with much enhanced potency (27) by structure-activity studies and molecular modeling techniques. Such structurally simplified and constrained Bn/GRP analogues would be of great value in elucidating the binding conformations of both agonists and antagonists and might also have improved pharmacological and pharmacokinetic properties.

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Probing Peptide Backbone Function in Bombesin

A REDUCED PEPTIDE BOND ANALOGUE WITH POTENT AND SPECIFIC RECEPTOR ANTAGONIST ACTIVITY*

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Each peptide bond CONH group in the most important COOH-*terminal* octapeptide region of [Leu^{14}]bombesin was replaced by a CH_2NH group using recently developed rapid solid-phase methods. The resulting analogues were then examined for amylase releasing activity in guinea pig pancreatic acini and for their ability to inhibit binding of [^{125}I-Tyr^4]bombesin to acinar cells. Replacement of the Trp^8-Ala^9, Gly^{11}-His^{12}, and His^{12}-Leu^{13} peptide bonds resulted in about 1000-, 200-, and 300-fold losses in both amylase releasing activity and binding affinity. The Val^{10}-Gly^{11} replacement, however, retained 30% potency relative to the parent peptide. Ala^9-Val^{10} and Leu^{13}-Leu^{14} bond replacement analogues exhibited no detectable amylase releasing activity but were still able to bind to acini with K_d values of 1060 and 60 nM, respectively (compared to 15 nM for [Leu^{14}]bombesin itself). Subsequently, both analogues were demonstrated to be competitive inhibitors of bombesin-stimulated amylase release with IC_{50} values of 937 and 35 nM, respectively. [Leu^{14}-ψ-CH_2NH-Leu^{13}]Bombesin exhibits a 100-fold improvement in binding affinity compared to previously reported bombesin receptor antagonists and showed no affinity for substance P receptors. It was also a potent inhibitor of bombesin-stimulated growth of murine Swiss 3T3 cells with an IC_{50} of 18 nM. In terms of a bombesin receptor-binding conformation, these results may aid in the delineation of intramolecular hydrogen-bonding points and the eventual design of improved, conformationally restricted analogues.

There has been considerable interest in the design and development of competitive bombesin receptor antagonists as possible antimitotic agents since the discovery that bombesin (*p*Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) and related peptides (1) act as potent autocrine growth factors in human small cell lung carcinoma systems *in vitro* and *in vivo* (2, 3). These cells also contain high levels of bombesin immunoreactivity (4) and high affinity receptors for the peptide (5, 6).

There have been two published reports concerning peptide analogues capable of blocking the actions of bombesin. The first of these (7) described the ability of a substance P receptor antagonist to prevent bombesin-stimulated pancreatic amylase release with an IC_{50} in the millimolar range. The second (8) report concerned a bombesin analogue in which D-Phe replacement for His^{12} resulted in a competitive antagonist. Although this latter compound was specific for pancreatic bombesin receptors, it also had a relatively high IC_{50} of 4 mM.

In the present paper, rather than using the classical side chain modification strategies, we have adopted a more unusual peptide backbone modification approach to bombesin analogue design and antagonist discovery. This was prompted by a recent report (9) in which the tetragastrin pseudopeptide, *t*-butoxycarbonyl-Trp-Leu-ψ[CH_2NH]-Asp-Phe-NH_2, was found to be an effective gastrin receptor antagonist and our own work (10) on ψ-CH_2NH pseudooctapeptide somatostatin analogues which were helpful in examining potential peptide bond involvement in intramolecular hydrogen bonding and peptide conformation. Although the reduced peptide bond is only one of many possible alternatives (11) for altering the CONH linkage, it also has the advantage of being easily incorporated (12) by reductive alkylation with sodium cyanoborohydride and the appropriate protected amino acid aldehyde during the rapid solid-phase synthesis of a peptide. Synthetic work was concentrated on the COOH-terminal half of the bombesin peptide chain which earlier structure-activity studies (13) indicate to be primarily responsible for receptor binding and triggering of the biological signal. To eliminate the readily oxidized Met^{14} residue, the analogues described here were based on [Leu^{14}]bombesin which retains about 33% of the biological potency and binding affinity of bombesin itself (Table I).

EXPERIMENTAL PROCEDURES

Materials

Protected amino acids and other synthetic reagents were obtained from Advanced ChemTech, Louisville, KY. NIH strain guinea pigs (175–225 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health. HEPES[1] was from Boehringer Mannheim; purified collagenase (type CLSPA, 440 units/mg) from Worthington; sodium borate, soybean trypsin inhibitor, carbamylcholine, theophylline, bacitracin, and 8-bromo-cAMP from Sigma; essential vitamin mixture (100× concentrated) from Microbiological Associates; glutamine and gastrin-I(2–17) from Research Plus; ^{125}I-labeled *N*-succinimydyl-3-(4-hydroxyphenyl)propionate (1500 Ci/mmol) and Na^{125}I from Amersham Corp.; [^{3}H]thymidine from Du Pont-New England Nuclear; Phadebas amylase test reagent from Pharmacia LKB Biotechnology Inc.; bovine plasma albumin (Fraction V) from Miles Laboratories; A23187 from

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[1] The abbreviations used are: HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ^{125}I-BH-SP, ^{125}I-labeled Bolton-Hunter-substance P; LH-RH, luteinizing hormone-releasing hormone.

Behring Diagnostics; vasoactive intestinal polypeptide and substance P from Peninsula Laboratories. COOH-terminal octapeptide of cholecystokinin was a gift from M. Ondetti, Squibb Institute for Biomedical Research, Princeton, NJ.

The standard incubation solution used in experiments involving pancreatic acini contained 24.5 mM HEPES (pH 7.4), 5 mM, NaCl, 2.5 mM, KCl, 5 mM NaH_2PO_4, 5 mM Na pyruvate, 5 mM, Na fumarate, 5 mM Na glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5 mM theophylline, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) amino acid mixture, and 1% (v/v) essential vitamin mixture. The incubation solution was equilibrated with 100% O_2 and all incubations were carried out with O_2 as the gas phase.

Methods

Preparation of Peptides—Solid-phase syntheses, including introduction of each reduced peptide bond, were carried out by the standard methods recently described by Sasaki and Coy (12). The crude hydrogen fluoride-cleaved peptides were purified on a column (2.5 × 90 cm) of Sephadex G-25 which was eluted with 2 M acetic acid, followed by preparative medium pressure chromatography on a column (1.5 × 45 cm) of Vydac C_{18} silica (15–20 μm) which was eluted with a linear gradient of 15–55% acetonitrile in 0.1% trifluoroacetic acid using an Eldex Chromatrol gradient controller (flow rate 1 ml/min). Analogues were further purified by re-chromatography on the same column with slight modifications to the gradient conditions when necessary. Homogeneity of the peptides was assessed by thin layer chromatography and analytical reverse-phase high pressure liquid chromatography, and purity was 97% or higher. Amino acid analysis gave the expected amino acid ratios. The presence of the reduced peptide bond was demonstrated by fast atom bombardment mass spectrometry. Each of the 6 analogues gave good recovery of the molecular ion corresponding to the calculated molecular mass of 1587.

Tissue Preparation—Dispersed acini from guinea pig pancreas were prepared as described previously (14).

Amylase Release—Dispersed acini from one guinea pig pancreas were suspended in 150 ml of standard incubation solution and samples (250 μl) were incubated for 30 min at 37 °C. Amylase activity was determined by the method of Ceska *et al.* (15, 16) using the Phadebas reagent. Amylase release was calculated as the percentage of amylase activity in the acini at the beginning of the incubation that was released into the extracellular medium during the incubation.

Binding of [^{125}I-Tyr^4]Bombesin—[^{125}I-Tyr^4]Bombesin (2000 Ci/mmol) was prepared using a modification (17) of the chloramine-T method of Hunter and Greenwood (18). [^{125}I-Tyr^4]Bombesin was separated from ^{125}I using a Sep-Pak and separated from unlabeled peptide by reverse-phase high pressure liquid chromatography on a column (0.46 × 25 cm) of μBondapak C_{18}. The column was eluted isocratically with acetonitrile (22.5%) and triethylammonium phosphate (0.25 M, pH 3.5) (77.5%) at a flow rate of 1 ml/min. Incubations contained 0.05 nM [^{125}I-Tyr^4]bombesin and were for 30 min at 37 °C. Nonsaturable binding of [^{125}I-Tyr^4]bombesin was the amount of radioactivity associated with the acini when incubation contained 0.05 nM [^{125}I-Tyr^4]bombesin plus 1 mM bombesin. All values shown are for saturable binding, *i.e.* binding measured with [^{125}I-Tyr^4]bombesin alone (total) minus binding measured in the presence of 1 mM unlabeled bombesin (nonsaturable binding). Nonsaturable binding was <20% of total binding in all experiments.

Binding of ^{125}I-Bolton-Hunter-Substance P (^{125}I-BH-SP)—^{125}I-BH-SP (1500 Ci/mmol) was prepared using a modification (19) of the method of Bolton and Hunter (20) and purified by reverse-phase high pressure liquid chromatography on a C_{18} column (21). Binding of ^{125}I-BH-SP to dispersed pancreatic acini was measured as described previously (19). Nonsaturable binding of ^{125}I-BH-SP was the amount of radioactivity associated with the acini when the incubation contained 0.06 nM ^{125}I-BH-SP plus 1 mM unlabeled substance P. All values given are for saturable binding, *i.e.* binding measured with ^{125}I-BH-SP alone (total) minus binding measured with 1 mM unlabeled substance P (nonsaturable). Nonsaturable binding was <20% of total binding in all experiments.

Growth of Swiss 3T3 Fibroblasts—Stock cultures of Swiss 3T3 cells (American Type Culture Collection CCL 92) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in an atmosphere of 10% CO_2, 90% air at 37 °C. The cells were seeded into 24-well cluster trays and used 4 days after the last change of medium. The cells were arrested in the G_1/G_0 phase of the cell cycle by changing to serum-free medium prior to thymidine uptake determinations.

Assays of DNA Synthesis—3T3 cells were washed twice with 1-ml aliquots of medium (without serum) and then incubated with medium, 0.5 mM [^{3}H]thymidine (20 Ci/mmol), bombesin (1 nM), and several concentrations of bombesin analogue in a final volume of 0.5 ml. After 28 h at 37 °C, [^{3}H]thymidine incorporation into acid-insoluble pools was then determined. Cells were washed twice with ice-cold 0.9% saline (1-ml aliquots) and acid-soluble radioactivity was removed by a 30-min (4°) incubation with 5% trichloroacetic acid. The cultures were washed once with 95% ethanol (1 ml) and solubilized by a 30-min incubation with 0.1 N NaOH. The solubilized material was measured for radioactivity on the scintillation counter.

RESULTS

We were interested in quantitating both the agonist and potential antagonist activity of the 6 analogues which were synthesized. They were, therefore, initially examined for stimulating effects on pancreatic amylase release, which is a major biological activity of bombesin peptides, and the dose-response curves obtained are shown in Fig. 1 in comparison to bombesin and [Leu^{14}]bombesin standards. EC_{50} values calculated from half-maximal stimulation concentration are given in Table I. Only [Val^{10}-ψ-CH_2NH-Gly^{11},Leu^{14}]bombesin retained high potency, being about three times less active than [Leu^{14}]bombesin itself. Analogues with 11–12, 12–13, and, particularly, 8–9 peptide bond replacement were several orders of magnitude less potent, but were full agonists. In contrast, 9–10 and 13–14 bond replacement completely destroyed detectable amylase releasing activity. The analogues were then tested for their abilities to inhibit binding of [^{125}I-Tyr^4]bombesin to pancreatic acini and inhibition curves are shown in Fig. 2 with calculated K_d values given in Table I. All analogues displayed affinities that correlated completely with their biopotencies with the exception of the 9–10 and 13–14 replacement peptides which were able to bind with K_d values of 1060 and 60 nM, respectively, despite having no amylase releasing activity.

The 9–10 and 13–14 replacement peptides were tested for inhibition of the amylase release produced by a 0.2 nM dose of bombesin (Fig. 3). Both gave concentration-dependent inhibition of the activity of bombesin and the calculated IC_{50} values were 937 ± 8 and 35 ± 7 nM, respectively. Finally, the antagonists were examined for their specificity towards bom-

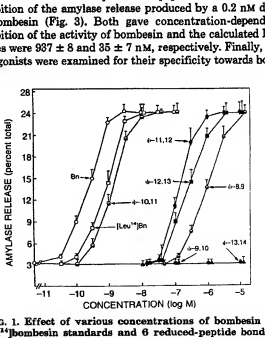

FIG. 1. Effect of various concentrations of bombesin and [Leu^{14}]bombesin standards and 6 reduced-peptide bond replacement analogues on amylase release from dispersed guinea pig pancreatic acinar cells under conditions described in the text. Values are the means from five experiments ± standard error.

TABLE I

Comparison of the ability of bombesin and [Leu[14]]peptide bond replacement analogues to stimulate dispersed acinar amylase release and displace [^{125}I-Tyr^{4}]bombesin from intact cells

K_d values for binding of the analogues were calculated by the method of Cheng and Prusoff (28). The K_d value for bombesin was determined by Scatchard analysis. EC_{50} and IC_{50} values are from data shown in Figs. 2 and 3 and represent concentrations of peptide causing half-maximal amylase release or half-maximal inhibition of 0.2 nM bombesin-stimulated release, respectively. Each value is the mean ± S.E. of five experiments.

Bond replaced	Amylase release (EC_{50})	Binding (K_d)
	nM	
8 9 10 11 12 13 14 -Trp-Ala-Val-Gly-His-Leu-Met-NH_2	0.2 ± 0.1	4.4 ± 0.6
Leu	0.8 ± 0.3	15.1 ± 2.9
~[a]	1060.5 ± 14.5	15500.6 ± 2040
~	ND[c]	1060.7 ± 140.8
~	2.1 ± 0.3	38.9 ± 5.9
~	140.6 ± 20.5	2410.8 ± 154.5
~	251.8 ± 36.6	4512.6 ± 1132.3
~	ND[c]	59.6 ± 5.8

[a] ~, Peptide bond replaced.
[b] Antagonist, IC_{50} = 937 ± 8 nM.
[c] Antagonist, IC_{50} = 35 ± 7 nM (see Fig. 3).

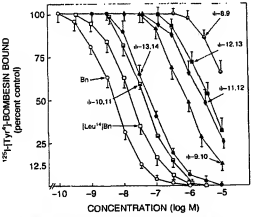

FIG. 2. **Displacement of ^{125}I-labeled bombesin from intact guinea pig pancreatic acinar cells by various concentrations of bombesin, [Leu^{14}]bombesin, and 6 peptide bond replacement analogues under conditions described in the text.** Values are the means from five experiments ± standard error.

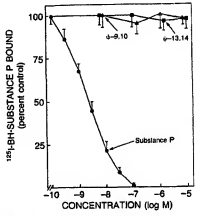

FIG. 4. **Displacement of ^{125}I-labeled Bolton-Hunter-substance P from guinea pig pancreatic acinar cells by a substance P standard and [Leu^{13}-ψ-CH$_2$NH-Leu^{14}]- and [Ala^{9}-ψ-CH$_2$NH-Val^{10}]bombesin under conditions described in the text.** Values are the means from five experiments ± standard error.

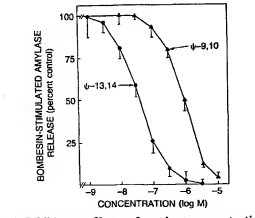

FIG. 3. **Inhibitory effects of various concentrations of [Leu^{13}-ψ-CH$_2$NH-Leu^{14}]- and [Ala^{9}-ψ-CH$_2$NH-Val^{10}]bombesin on guinea pig pancreatic acinar amylase release stimulated by 0.2 nM bombesin under conditions described in the text.** Values are the means from five experiments ± standard error.

besin receptors. Importantly, no inhibition of the binding of ^{125}I-labeled substance P could be achieved at concentrations up to 10 mM (Fig. 4). No inhibition of amylase release stimulated by substance P, cholecystokinin 8, vasoactive intestinal polypeptide, 8-bromo-cAMP, or A21837 was evident at the concentrations tested (Table II). The dose-response curve for bombesin-stimulated amylase release was shifted in a parallel fashion to the right by increasing concentrations of either the 13–14 (data not shown) or 9–10 bond replacement peptide. Schild plots for both peptides demonstrated a slope not significantly different from unity with a K_d of 22 ± 6 nM for the 9–10 bond replacement analogue.

The antagonist activity of [Leu^{13}-ψ-CH$_2$NH-Leu^{14}]bombesin was also examined in a totally different biological system, murine Swiss 3T3 fibroblast cells, the growth of which is stimulated by bombesin agonists (22) and which are known to contain bombesin receptors (23). Excellent inhibition of bombesin-stimulated growth was demonstrated and data from three experiments based on [^{3}H]thymidine incorporation are shown in Fig. 5. An average IC_{50} of 18 nM (Table III) was obtained from these experiments, which agrees very well with that derived from the acinar cell system. For comparison, a

TABLE II

Effect of [Leu^{13}-ψ-CH$_2$NH-Leu^{14}]- and [Ala^{8}-ψ-CH$_2$NH-Val^{10}, Leu^{14}]bombesin on guinea pig pancreatic acinar amylase release stimulated by various agents

Secretagogue	Amylase release		
	Alone	+0.3 mM 13–14 analogue	+10 mM 9–10 analogue
		% *total*[a]	
None	3.9 ± 0.7	4.1 ± 0.3	4.0 ± 0.2
Bombesin (0.2 nM)	17.1 ± 2.3	4.3 ± 0.3[b]	5.1 ± 1.0[b]
Substance P (3 nM)	10.3 ± 1.1	10.8 ± 0.3	11.2 ± 0.7
Cholecystokinin 8 (0.1 nM)	35.6 ± 2.2	33.9 ± 2.1	36.7 ± 3.0
Carbachol (10 mM)	28.4 ± 2.2	28.0 ± 2.9	30.0 ± 3.6
Vasoactive intestinal polypeptide (0.1 nM)	20.2 ± 2.4	20.4 ± 0.9	22.1 ± 1.1
8-Bromo-cAMP (1 mM)	24.0 ± 2.1	23.8 ± 2.5	25.1 ± 7.3
A21387 (3 mM)	13.2 ± 1.9	12.3 ± 0.3	13.0 ± 1.3

[a] Results are the means ± S.E. from five separate experiments.
[b] $p < 0.01$ compared to bombesin alone.

TABLE III

Comparison of the effectiveness of inhibition of 3T3 cell growth by two bombesin antagonists and a substance P antagonist

Peptide	IC_{50}
	nM
[D-Phe^{12}]Bombesin	>5000
[D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}] Substance P	2900
[Leu^{13}-ψ-CH$_2$NH-Leu^{14}]Bombesin	18 ± 12[a]

[a] Calculated from the data shown in Fig. 5.

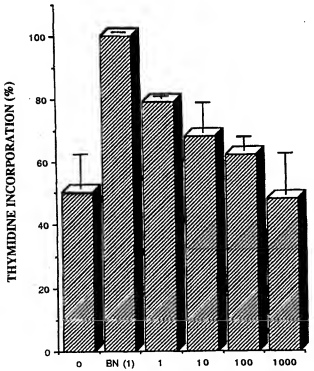

FIG. 5. **[Leu^{13}-ψ-CH$_2$NH-Leu^{14}]Bombesin inhibition of bombesin-stimulated [^{3}H]thymidine incorporation into murine Swiss 3T3 cells in culture.** Values are the means from three experiments ± standard error.

substance P receptor antagonist exhibited an IC_{50} of 2600 nM and our previous [D-Phe^{12}]bombesin antagonist was not effective at concentrations up to 5000 nM.

DISCUSSION

Although modifications to a peptide bond have long been considered an interesting approach to structure-activity relationships, it was not until recently that the chemistry for introducing one of them, the CH_2NH group, was simplified by adapting it to rapid solid-phase methods (12). Therefore, we are only just beginning to build a sufficiently large data base for this type of analogue with which to eventually derive some indication of what can be expected in terms of effects on biological activity generally. Thus far, reduced peptide bond somatostatin (10), gastrin (9), and bombesin analogues have not yielded any compounds with increased biopotency caused by increased receptor affinity. Likewise, in a reduced peptide bond series of luteinizing hormone-releasing hormone (LH-RH) antagonists (24), no analogues were found with improved antagonist activity. On the other hand, both the gastrin and the present bombesin studies resulted in the discovery of more than one antagonist analogue in each case. It is tempting to conclude, therefore, that this may be the design approach of choice for antagonist discovery.

Generally, the tendency for loss of potency in a peptide agonist series is probably explained by the profound effects which elimination of a peptide bond CO group will have on conformation due to both loss of a potential intramolecular hydrogen-bonding point and increased rotation about the C–N bond. In a folded peptide conformation, hydrogen bonding is a prime factor stabilizing the structure and in our previously reported somatostatin octapeptide series (10), for which much physicochemical data existed, replacement of hydrogen bonds not involved in this process tended to retain the most activity.

With [Leu^{13}-ψ-CH$_2$NH-Leu^{14}]bombesin it is entirely possible that the 13–14 peptide bond CO group, although clearly not necessary for binding, is directly responsible for triggering the receptor response. However, this does not account for the antagonist activity also produced by 9–10 bond replacement. We suggest that another explanation could reside in destabilization of a folded, extensively hydrogen-bonded conformation similar to those present in somatostatin analogues (25), LH-RH (26), and several other peptides. Fig. 6 attempts to show this. We have placed the beginning of a β-turn at Val^{10} so that Gly^{11} occupies a pivotal position. The rest of the chain, modeled on the known solution conformation of conformationally restricted somatostatin octapeptides (25), is arranged in the form of an antiparallel β-pleated sheet. It should be

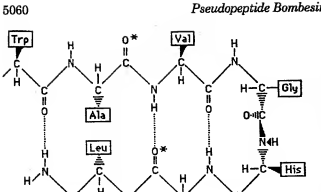

FIG. 6. **Possible conformation for the COOH-terminal octapeptide region of [Leu^{14}]bombesin with a type II′ β-bend involving the Val-Gly-His-Leu tetrapeptide.** Carbonyl groups (*) which produce antagonists when replaced by CH_2 and putative intramolecular hydrogen bonding interactions along the chain are shown.

noted that Gly^{11} in bombesin can be replaced by D-Ala with complete retention of activity, which prompted Rivier and Brown (13) and, more recently, Erne and Schwyzer (27) to discuss a similar β-bend. Also, the β-bends in somatostatin and LH-RH are similarly characterized by residues (Trp and Gly in positions 8 and 6, respectively) which can be replaced by D-amino acids with, in these cases, actual improvements in potency. In the β-pleated sheet area of the model, hydrogen bonding between the Leu^{13}-Leu^{14}-CO group and the Ala^{2}-Val^{10}-NH group assumes some importance and we propose that its destruction in the 13–14 analogue could result in a conformational shift responsible for loss of biological activity. Additionally, the 9–10 NH group would be adjacent to the Ala^{2}-CO group which, when replaced by CH_2 also results in an antagonist. It is thus conceivable that the same hydrogen bond could also be inhibited by the 9–10 peptide bond replacement since bond angles and rotational freedom would all be significantly affected. Also noteworthy in relation to this model are the loss of activity caused by replacement of the Trp^{8}-CO group which, could also be involved in another hydrogen bond, and the previously described importance of the COOH-terminal amide (13) which would also contribute to the same interaction. There is also loss of activity, although much less dramatic, associated with the Val^{10}-CO replacement which constitutes part of the hydrogen bond integral to the β-bend. It should be emphasized that no direct physicochemical evidence from solution studies exists to support this hypothesis and, indeed, a recent infrared spectroscopic study of bombesin in phospholipid bilayer membranes (27) points to a α-helical structure in this environment. However, as Erne and Schwyzer (27) point out, the helical structure of the membrane-bound peptide could actually facilitate a second conformational transition caused by interaction with the receptor which could well involve the proposed β-turn and hydrogen bonding points. In any event, the model does provide a useful starting point for the design of additional, conformationally covalently restricted linear and cyclic analogues in the future.

An additional advantage to this type of analogue design strategy appears to lie in the absence of multiple side chain modifications which are so often needed for development of potent antagonists by standard approaches. This can often result in the introduction of undesirable properties, such as the loss of specificity with the substance P antagonists (7), introduction of enhanced histamine releasing activity with the LH-RH antagonists (28), and the poor solubility properties of the [D-Phe^{12}]bombesins. In contrast, both of the present antagonists exhibited physical properties almost identical to bombesin and thus far both appear to be highly specific for bombesin receptors.

It is encouraging that the high antagonist activity of [Leu^{13}-ψ-CH$_2$NH-Leu^{14}]bombesin extended to an assay system examining bombesin-stimulated cellular growth where it is about 200 times more potent than the substance P inhibitor spantide, which is the only other compound reported capable of blocking the actions of bombesin in the 3T3 cell system. This indicates that there are no significant differences in receptor recognition requirements between acinar and 3T3 cells and suggests that the probability of this antagonist inhibiting bombesin-stimulated growth of human small cell lung carcinoma strains should be quite good. The development of a bombesin receptor antagonist with useful therapeutic properties may require additional synthetic work aimed at improving receptor affinity even further and particularly at improving *in vivo* pharmacokinetic properties. The present compound offers an excellent lead structure for this type of research.

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Bombesin/Gastrin-Releasing Peptide Receptor: A Potential Target for Antibody-Mediated Therapy of Small Cell Lung Cancer

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ABSTRACT

Purpose: **Bombesin/gastrin releasing peptide (BN/GRP) is a growth factor for small cell lung cancer (SCLC). The receptor (R) for BN/GRP is overexpressed on SCLC cells and other solid tumors. BN/GRP and its receptor form an autocrine loop to promote tumor growth. We developed a novel immunotherapeutic approach targeting cell surface BN/GRP-R on SCLC cells and an immune trigger molecule on host immune effector cells to direct immune effector cells to SCLC cells and mediate targeted cancer cell destruction. Targeted immunotherapy combined with chemotherapy enhanced cell killing.**

Experimental Design: **We designed a synthetic BN/GRP antagonist (Antag 2) and constructed a bispecific molecule (BsMol), H22xAntag 2 (humanized monoclonal antibody) for FcγRI. We tested the binding of the BsMol to several SCLC cell lines, its ability to mediate cytotoxicity of SCLC by IFN-γ-activated human monocytes with chemotherapy, and BsMol-mediated immunotherapy in an animal model of SCLC human xenograft.**

Results: **Common chemotherapy (cisplatin, etoposide, and paclitaxel) inhibited thymidine uptake into SCLC cells in a dose-dependent pattern. Antibody-dependent cellular cytotoxicity mediated by the BsMol inhibited thymidine uptake into SCLC cells and was largely dependent on E:T cell ratio. When SCLC cells were treated with antibody-dependent cellular cytotoxicity followed by exposure to chemotherapy agents an additional 25–40% inhibition of thymidine uptake into SCLC cells was observed consistently. With BsMol and IFN-γ-activated human monocytes, tumor burdens were reduced significantly in immunodeficient mice bearing human SCLC xenografts.**

Conclusions: **Combined chemotherapy and immunotherapy targeting BN/GRP-R with a BsMol significantly enhances targeted SCLC cell killing.**

INTRODUCTION

Lung cancer is the second most common malignancy in the United States. As estimated by the American Cancer Society, there will be 171,900 new cases and 157,200 deaths from lung cancer in 2003 (1). SCLC[2] accounts for 15–25% of all lung cancers. Although SCLC is sensitive to both chemotherapy and radiotherapy, the duration of response is usually short-lived. The majority of SCLC patients die from progressive disease.

Human GRP is a mammalian analogue of BN, initially discovered in the frog (2). BN/GRP is produced by majority of SCLC cell lines and binds to BN/GRP-R on their cell surfaces to form an autocrine loop to promote tumor growth (3). Interruption of this autocrine loop between BN/GRP and BN/GRP-R results in the inhibition of SCLC growth *in vitro* as well as *in vivo* (4, 5). There are three subtypes of BN/GRP-R, namely GRP-R, neuromedin B receptor, and BN receptor subtype 3 (6–9). Both BN and GRP bind to three subtypes of receptor with variable affinity. BN/GRP-R is coupled with G protein, which activates multiple signal transduction pathways on the binding of BN/GRP and results in cell proliferation (10–13).

In a cancer-bearing patient, the host immune system has become compromised and cannot mount an effective immune response to the growing tumor. Several approaches have been developed to break such immune incompetence, by activating certain trigger molecules on immune effector cells, by activating costimulatory pathways in T cells, or by eliciting a host immune response with a tumor vaccine. The Fc receptors for IgG (FcγRI), expressed on monocytes, macrophages, and neutrophils, are one of few molecules capable of mediating ADCC. FcγRI is a potent cytotoxic trigger molecule activated by a number of cytokines including IFN-γ, granulocyte/monocyte-colony-stimulating factor, and granulocyte-colony-stimulating factor (14). Targeting this receptor has the potential to recruit large numbers of immune effector cells and to redirect their cytotoxic activities toward cancer cells.

Growth factor receptors on tumor cell surfaces are ideal targets for immunotherapy. To our knowledge, there is no mAb directed to human GRP-R. We hypothesized that a BsMol could

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Dr. Ball owns stock in Medarex, the provider of the H22 antibody used in this study.
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[2] The abbreviations used are: SCLC, small cell lung cancer; R, receptor; BN, bombesin; GRP, gastrin releasing peptide; ADCC, antibody-dependent, cell-mediated cytotoxicity; mAb, monoclonal antibody; BsMol, bispecific molecule.

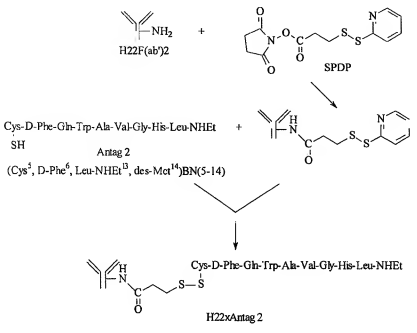

Fig. 1 Schema of the chemical conjugation of the BsMol. H22 was reacted with a cross-linker SPDP to form a 2-pyridyl disulfide-activated antibody. The final conjugation of H22xAntag 2 was achieved by mixing the cysteine-containing Antag. 2 with the antibody.

be constructed to target GRP-R on tumor cell surface and a cytotoxic trigger molecule on immune effector cells to activate ADCC (15). We constructed a BsMol consisting of a synthetic BN/GRP peptide and a mAb directed to FcγRI. The BsMol could bind to SCLC cells and mediate specific lysis of SCLC cells *in vitro* (16, 17). Several clinical trials targeting FcγRI have been reported with encouraging results. A bispecific antibody composed of anti-FcγRI and anti-HER2 antibody, MDX-210, was evaluated in patients with advanced breast and ovarian cancer. After infusion, cytokine release was documented and tumor regression in patients was observed (18, 19). In a Phase II trial, patients with advanced prostate cancer received multiple doses of MDX-210 in combination with granulocyte/monocyte-colony-stimulating factor. Seven patients (35%) had >50% decrease in PSA levels after treatment (20). Infusion of BsAb consisting of anti-FcγRI and anti-CD33 antibody was evaluated in a Phase I trial in patients with relapsed or refractory acute myeloid leukemia (21). In those trials, activation of host immune responses was documented; most patients tolerated the infusion well.

There is ample laboratory and clinical evidence that combining immunotherapy with conventional chemotherapy results in either additive or synergistic effects on tumor cell killing (22–24). Rituximab (anti-CD20 mAb) in combination with chemotherapy has become a standard treatment for CD20-positive lymphoma (25). Herceptin (anti-HER2 mAb) combined with various chemotherapy agents has been used to treat metastatic breast cancer (26, 27). The mechanism for the enhanced effects of cancer cell killing between chemotherapy agents and mAb is unclear. A simple explanation is that there is additive cell killing mediated by two different agents acting on different targets. Evidence suggests that an antibody targeting a growth factor receptor significantly enhances the effect of chemotherapy by a mechanism called receptor-enhanced chemosensitivity (22, 23). Conversely, chemotherapy may enhance the effect of growth inhibition by an antibody against a growth factor receptor (28–30).

We chose to simplify the steps of chemical conjugation of a BsMol targeting BN/GRP-R, to study the effect of targeted immunotherapy of xenografted human SCLC in a murine model, and to evaluate the effect of combined immunotherapy and chemotherapy on SCLC cells.

MATERIALS AND METHODS

Construction of BsMol. Detailed chemical construction of a BsMol has been published previously (16, 17). To simplify the construction process, we added a cysteine residue to the NH_2-terminal of a BN antagonist, (D-Phe^6, Leu-NHEt^{13}, and des-Met^{14}) BN(6–14) to create a free sulfhydryl group (31). The peptide (Cys^5, D-Phe^6, Leu-NHEt^{13}, and des-Met^{14}) BN(5–14), named Antag 2, was custom synthesized (BACHEM, Torrance, CA). The quantity of free sulfhydryl group on Antag 2 was determined by an Ellman's test. H22, a humanized mAb F(ab')$_2$ fragment against FcγRI (Medarex, Inc., Princeton, NJ), was incubated with a cross-linker, N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), to form a 2-pyridyl disulfide-activated antibody (Fig. 1). Unreacted SPDP was removed by centrifugation through a size-exclusion filter. Antag 2 was added to the reactive antibody in a final molar ratio of 10:1. After 18 h of incubation, unreacted Antag 2 was removed by centrifugation through a size-exclusion filter. Concentration of the synthetic BsMol, H22xAntag 2, was determined by DC Protein Assay (Bio-Rad Laboratory, Hercules, CA).

Cell Culture. SCLC cell lines H345 and H69 were purchased from American Type Culture Collection (Rockville, MD). DMS273 was a SCLC cell line established from pleural effusion of a patient (32, 33). DMS273 was provided to us by

Drs. Olive S. Pettengill and George D. Sorenson of Dartmouth Medical School (Lebanon, NH). All of the cell lines were maintained in serum-free RPMI 1640 medium containing 1×10^{-8} M hydrocortisone, 5 μg/ml of insulin, 10 μg/ml of transferrin, 1×10^{-8} M β-estradiol, and 3×10^{-8} M selenium (HITES medium) (all purchased from Sigma Chemical Co., St. Louis, MO).

Colony Assay. SCLC cells (5×10^3) were suspended in 1 ml of HITES medium containing 0.3% agarose. Cells were plated over a base layer of 1 ml of HITES medium of 0.5% agarose. BN, Antag 2, and the BsMol were added at different concentrations. Cells were incubated for 14 days; colonies were counted under a reversed-phase light microscope. A colony was defined as a distinct aggregate of >50 cells.

Binding Study by Flow Cytometry Analysis. Cells were washed with PBS containing 0.1% BSA and 0.1% sodium azide (PBA). Cells (3×10^5) in 100 μl of PBA were incubated with different concentrations of BsMol for 1 h at 4°C. After incubation, the cells were washed twice and incubated with goat $F(ab')_2$ antihuman IgG FITC (Jackson ImmunoResearch Inc., West Grove, PA) for 30 min at 4°C, then fixed in 1% paraformaldehyde. To test the specificity of the binding, the cells were incubated with Antag 2 or BN at different concentrations for 30 min before adding the BsMol. Unconjugated H22 and angiotensin (BACHEM), a structurally unrelated peptide, were used as negative controls. Samples were analyzed by FACScan using Cellquest software (Becton-Dickinson Immunosystem, San Jose, CA).

Western Blot Analysis. Freshly cultured SCLC cells and peripheral blood lymphocytes were washed with PBS, lysed in the triple-detergent lysis buffer [50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (pH 8.0)]. The cell lysate at 20 μg of protein per lane was loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to a nitrocellulose membrane. The membrane was incubated first with 5% nonfat milk for 2 h and then with BsMol for 2 h at 4°C. Unconjugated H22 was used as negative control. After incubation, the membrane was washed and a peroxidase-conjugated goat $F(ab')_2$ antihuman IgG (Jackson ImmunoResearch) was added to a final dilution of 1:1000. After 1 h of incubation, the membrane was washed extensively and incubated with a chemiluminescent reagent according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). The signal was detected by exposing the membrane to a Kodak film.

Thymidine Incorporation Assay. Cisplatin and paclitaxel were obtained from Bristol Myers-Squibb Company (Princeton, NJ). Etoposide was obtained from Bedford Laboratories (Bedford, OH). Growth inhibition of tumor cells was measured by a [^{3}H]thymidine incorporation assay. Cells in complete medium were seeded ($2.5–5 \times 10^3$/well) onto a 96-well plate. After 48 h of culture, individual drugs were added at various concentrations. Cells were incubated with each agent continuously for 72 h. [^{3}H]Thymidine was added during the last 8 h of incubation.

Preparation of Immune Effector Cells. Mononuclear cells were separated from peripheral blood of healthy donors using Ficoll-Hypaque density gradient centrifugation. After 2 h of incubation, nonadherent cells were removed. Adherent cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 150 units/ml of IFN-γ overnight. Cells were detached from the culture dish by incubating with HBSS medium and 2 mM EDTA for 5 min. The phenotype of the cells and the expression of FcγRI were determined by flow cytometry. The cell preparation contained 80–90% monocytes.

ADCC and Combined Treatment. Target cells (T) at $2.5–5 \times 10^3$/well were seeded onto a 96-well plate. IFN-γ-activated monocytes (effector cells) were added at E:T ratios of 30:1, 15:1, and 7.5:1. The BsMol or unconjugated H22 was added in a final concentration of 1 μg/ml. To evaluate the specific killing mediated by BsMol, free Antag2 was incubated with target cells at 5 μg/ml, and unconjugated H22 was incubated with effector cells at 10 μg/ml for 15 min before adding BsMol. The cell mixture and control cells (tumor cells alone) were cultured for 48 h. Individual chemotherapy agent, cisplatin, etoposide, or paclitaxel, was added and cultured continuously for another 72 h. [^{3}H]Thymidine was added during the last 8 h of culture. Cells were harvested by a Tomtec cell harvester (Perkin-Elmer, Downers Grove, IL) and counted in a liquid scintillation counter. All of the assays were performed in triplicate. Thymidine incorporation was calculated as: (experimental cpm − monocytes cpm)/tumor cells alone cpm × 100%. The inhibition of thymidine incorporation was calculated as: [1-(experimental cpm − monocytes cpm)/tumor cells alone cpm)] × 100%.

***In Vivo* Study.** Six to 8-week-old NOD.CB17-Prkdc SCID mice were (Jackson Laboratory, Bar Harbor, ME) maintained in a pathogen-free facility. In each experiment, mice were divided into three groups (tumor cells alone, immunotherapy with unconjugated H22, and immunotherapy with BsMol) with 3–4 mice in each group. They were irradiated with 300cGy immediately before the injection of 1×10^6 DMS273 cells i.p. on day 1. IFN-γ-activated human monocytes (1×10^7) were mixed with H22 or BsMol (50 μg/mouse) and injected i.p. on days 3 and 6. All of the mice were sacrificed on day 28. The peritoneal cavity was washed with 5 ml of normal saline twice, and the peritoneal exudate was drawn into the syringe and collected for flow cytometry analysis. The abdominal cavity was then dissected and carefully inspected. All of the visible tumor nodules were resected, weighed, and fixed in 10% buffered formalin solution. They were embedded in paraffin and sectioned at 5 μm for light microscopic examination. Tumor burden was determined by: (*a*) total weight of all resectable tumor nodules in the peritoneal cavity; and (*b*) percentage of human CD15/CD56 dual-positive cells in the peritoneal exudate. DMS273 expresses both CD15 and CD56, allowing the detection of remaining SCLC cells.

Statistics. The Student *t* test was used to compare two groups of samples. The significance level was determined when P was <0.05 by two-sided analysis. The results are presented as mean ± SD.

RESULTS

Specific Binding of the BsMol to SCLC Cell Lines. The BsMol bound to 60–80% of cells from three SCLC cell lines in a dose-dependent pattern (Fig. 2). This binding profile

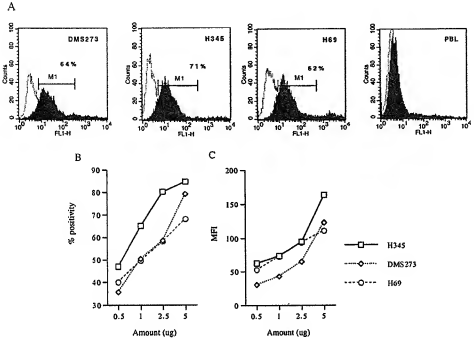

Fig. 2 Flow cytometry analysis of the BsMol binding to three SCLC cell lines. *A*, the *blank peak* was the negative control. The *solid peak* showed a positive staining of three SCLC cell lines with the BsMol, H22xAntag 2. Human PBL negative for BN/GRP-R expression was used as control. *B*, the percentage of positive cells increased with the amount of BsMol in a dose-dependent pattern. *C*, mean fluorescence intensity as an indirect measure of binding sites also increased with the amount of BsMol in a dose-dependent pattern.

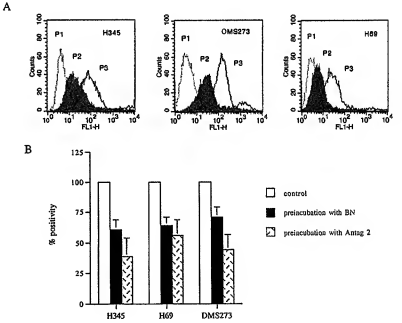

Fig. 3 Blocking of the BsMol binding to three SCLC cell lines. *A*, *P1*, cells incubated with unconjugated H22. *P2*, cells preincubated with free Antag 2 at 50 μM, then with BsMol. *P3*, cells incubated with BsMol. *B*, compared with BsMol, preincubation of cells with free Antag.2 or free BN blocked 45–60% and 30–40% of the binding.

was consistent with previous data (17), demonstrating that the addition of a cysteine residue at the NH_2-terminal of the known antagonist (D-Phe^6, Leu-NHEt^{13}, and des-Met^{14}) BN(6–14), had no adverse effect on the binding capacity. The mean fluorescence intensity, as an indirect estimation of binding sites, increased in a dose-responsive pattern. The unconjugated H22 alone did not bind to these cell lines and was used as a control. The BsMol did not bind to human peripheral blood lymphocytes. To determine the specificity of the binding to BN/GRP-R, cells were preincubated with Antag 2 (50 μM), BN (50 μM), angiotensin (50 μM), or unconjugated H22 (10 μg/ml). The binding of BsMol to SCLC cells was partially blocked by Antag 2 (45–60%) and BN (35–40%), but was not blocked by angiotensin and H22 (Fig. 3).

Fig. 4 Western blot analysis. The BsMol stained a single positive band with a molecular weight of M_r ~75,000. *Lane 1*, negative control (human lymphocytes). *Lane 2*, positive control (Swiss 3T3 cells). *Lane 3*, H69 cells. *Lane 4*, DMS 273 cells. *Lane 5*, H345 cells. *Lane 6*, molecular weight marker.

On Western blot analysis, the BsMol stained a single band from three SCLC cell lines with a molecular weight of M_r 75,000–80,000 (Fig. 4). Swiss 3T3 cells and human PBL were used as positive and negative control, respectively. The molecular weight of the detected protein was consistent with BN/GRP-R (6–8).

Effect of Antag 2 and the BsMol on Cell Proliferation. In the presence of BN, the number of SCLC colonies was increased. The growth stimulatory effect of BN was effectively blocked by the addition of Antag 2. The presence of the BsMol, H22xAntag 2 at 1–10 μg/ml, had no significant effect on the number of SCLC colonies (Fig. 5). This observation also confirms our hypothesis that the addition of a cysteine residual at the NH_2 terminus had no adverse effect on the biological function of the Antag 2.

Effect of Chemotherapy Agents on Thymidine Uptake into SCLC Cells. Inhibition of thymidine uptake into SCLC cells was dependent on the concentration of the chemotherapy agent. After continuous exposure of 72 h, 80–100% inhibition of thymidine uptake was achieved in all three of the SCLC cell lines. The sensitivity to chemotherapy agents varied. The IC_{50}, defined as the concentration resulting in 50% inhibition of thymidine uptake, was 5 nM, 4 nM, and 2.5 nM of paclitaxel for H345, DMS273, and H69 cells, respectively. The IC_{50} was 0.1 μM, 0.25 μM, and 0.5 μM of etoposide for H345, DMS273, and H69 cells, respectively. The IC_{50} was 0.5 μM of cisplatin for all three of the cell lines. Dose-response curves are shown in Fig. 6. To demonstrate additional inhibition of thymidine uptake from the addition of ADCC, we chose concentrations of each agent approximating IC_{20} to IC_{30} for subsequent experiments described later.

Effect of ADCC and Combined Treatment on SCLC Cells. Thymidine uptake into control cells incubated with medium alone for 5 days was defined as 100%. Inhibition of thymidine uptake by BsMol-mediated ADCC was dependent on E:T ratio. At E:T ratio of 30:1, 80–90% inhibition of thymidine uptake was achieved. At E:T ratio of 7.5:1, 20–40% inhibition was observed. When tumor cells were preincubated with free Antag 2 and monocytes preincubated with unconjugated H22 before adding BsMol, the inhibition of thymidine uptake was blocked by 50–70% in both DMS273 and H69 cells. When we combined ADCC with each chemotherapy agent at IC_{30}, an additional 25–40% inhibition was consistently observed compared with chemotherapy alone. A typical experiment with an E:T ratio of 7.5:1 is presented in Fig. 7. The activity of effector cells varied from individual donors, and the sensitivity of SCLC cells to individual donor cells also varied. The summary of four experiments using effector cells from four different donors is

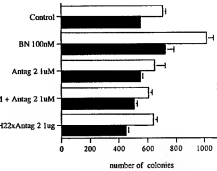

Fig. 5 Result of clonogenic assay of two SCLC cell lines. The presence of 100 nM BN significantly increased the number of colonies of both H69 and DMS 273 cells. The presence of Antag 2 or BsMol alone had no effect on colony numbers. However, the growth stimulatory effect of BN was blocked by the Antag 2 at 1 μM.

presented in Tables 1 and 2. For DMS273 cells, chemotherapy alone resulted in 10–30% inhibition, ADCC alone in 30–40% inhibition, and combined treatment in 60–80% inhibition of thymidine uptake. Compared with chemotherapy alone, a relative increase of 50–80% was observed. Compared with ADCC alone, a relative increase of 35–60% was observed. For H69 cells, chemotherapy, ADCC, and combined treatment resulted in 20–30%, 30–40%, and 50–70% inhibition of thymidine uptake, respectively. Compared with chemotherapy and ADCC alone, combined treatment resulted in a relative increase of 35–60% and 25–50% increase of inhibition. The observed increase of inhibition was highly statistically significant.

***In Vivo* Experiments.** All of the control mice injected i.p. with 1×10^6 DMS273 cells had tumor growth in the peritoneal cavity by day 28. The tumor weight ranged from 0.4 to 1.4 gram/mouse. Tumor nodules were usually well capsulated. Most mice developed obstructive jaundice, without distant metastases in the liver, spleen, gastrointestinal tract, kidney, or reproductive tissues. The total number of peritoneal exudate cells were $6.8 \pm 6.4 \times 10^6$, and 8.4 ± 2.3% of those cells were positive for human CD56/CD15. The timing and frequency of treatment were determined from preliminary experiments. One injection of BsMol and effector cells was not adequate for control tumor growth. The number of human monocytes injected was determined based on *in vitro* experiments; an E:T ratio of 10:1 was used. The results from three experiments are summarized in the Table 3. Eleven of 12 mice treated with human monocytes and unconjugated H22 had visible tumor growth. The tumor volume was not significantly different in the control mice. Mice treated with human monocytes and BsMol had significantly less tumor volume compared with the mice treated with human monocytes and H22 ($P = 0.001$, two-sided). Two of the 11 mice treated with human monocytes and BsMol had no macroscopic tumor in their peritoneal cavities.

DISCUSSION

The BN/GRP-R is expressed on malignant cells from patients with SCLC, as well as other cancers such as breast and prostate. Our novel targeting molecule is composed of a peptide

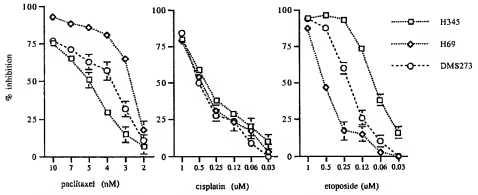

Fig. 6 Inhibition of thymidine uptake into SCLC cells by paclitaxel, cisplatin, and etoposide. SCLC cells were continuously exposed to individual chemotherapy agent for 72 h. Inhibition (80–100%) of thymidine uptake was observed with 10 nM of paclitaxel, 1 μM of cisplatin, and 1 μM of etoposide. The dose-response curve of each cell line for individual chemotherapy agent was slightly different. H69 cells were more sensitive to paclitaxel, and H345 cells were more sensitive to etoposide. The sensitivity to cisplatin was similar for three cell lines.

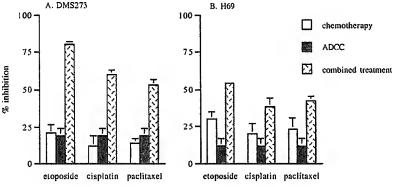

Fig. 7 Inhibition of thymidine uptake by chemotherapy, immunotherapy, and combined treatment. Chemotherapy alone resulted in 15–30% inhibition of thymidine uptake. ADCC with an E:T ratio of 7.5:1 resulted in 10–25% inhibition. With a combined treatment, 50–80% inhibition was observed for DMS273 cells, and 40–55% inhibition was observed for H69 cells.

Table 1 Cytotoxicity of DMS273 SCLC cells[a]

	Chemotherapy	ADCC	Combination	P^b
Etoposide	24 ± 14	36 ± 18	68 ± 9	0.005/0.03
Cisplatin	31 ± 11	36 ± 18	70 ± 4	0.003/0.03
Paclitaxel	10 ± 8	36 ± 18	59 ± 9	0.01/0.02

[a] Average percentage of cytotoxicity from four experiments.
[b] Chemotherapy *versus* combined therapy/ADCC *versus* combined therapy.

Table 2 Cytotoxicity of H69 SCLC cells[a]

	Chemotherapy	ADCC	Combination	P^b
Etoposide	22 ± 1	34 ± 15	52 ± 9	0.004/0.05
Cisplatin	28 ± 8	34 ± 15	54 ± 5	0.002/0.03
Paclitaxel	24 ± 4	34 ± 15	63 ± 12	0.01/0.03

[a] Average percentage of cytotoxicity from four experiments.
[b] Chemotherapy *versus* combined therapy/ADCC *versus* combined therapy.

ligand for the receptor and a mAb directed to the high-affinity Fc receptor expressed on mononuclear phagocytes and granulocyte-colony-stimulating factor-activated neutrophils. This BsMol is capable of mediating ADCC of SCLC cells. Combining the commonly used anticancer agents cisplatin, etoposide, and paclitaxel, and the BsMol targeting BN/GRP-R significantly inhibits SCLC cell growth *in vitro*. Targeted immunotherapy is dependent on E:T cell ratio *in vitro*. At a low E:T ratio, chemotherapy predominates. Monocyte activity varied among donors, as did the susceptibility of different SCLC cell lines to monocytes from different donors. Multiple variations made it difficult to determine whether combining chemotherapy with ADCC had an additive or a synergistic effect. However, a significant increase in tumor inhibition over ADCC alone was consistently observed when the E:T ratio was above 7.5:1.

We also tested the efficacy of BsMol-mediated ADCC alone in human SCLC xenografts in NOD/scid mice. In these "proof-of-principle" studies, the tumor volume of xenografted human SCLC was significantly reduced by administrating BsMol and human monocytes on days 3 and 6. Determining optimal conditions for this immunotherapy regarding timing, dosing of cells/antibody, and frequency requires additional study.

The simplified method reported here of BsMol construction is more suitable for large-scale production. We previously used a conjugation linker to create a sulfhydryl group on a

Table 3 Effect of immunotherapy on SCLC xenografts in SCID mice

Exp.	Groups	Mice	Body weight (g)	Tumor weight (g)	PEC[a]	P^b
1	Control	4	31.0 ± 1.2	0.71 ± 0.37	7.4 ± 4.3	0.006
	Monocytes + H22	4	28.5 ± 1.4	0.63 ± 0.22	5.1 ± 1.9	
	Monocytes + BsMol	4	28.9 ± 1.2	0.09 ± 0.05	2.2 ± 1.6	
2	Control	4	19.5 ± 2.5	1.07 ± 0.29	17.5 ± 12.7	0.14
	Monocytes + H22	4	22.5 ± 2.3	0.59 ± 0.55	5.2 ± 3.9	
	Monocytes + BsMol	4	23.6 ± 0.9	0.05 ± 0.05	2.7 ± 1.8	
3	Control	3[c]	29.0 ± 1.0	0.49 ± 0.09	12.9 ± 0.5	0.03
	Monocytes + H22	3	22.8 ± 1.0	0.82 ± 0.29	8.7 ± 6.6	
	Monocytes + BsMol	3	26.8 ± 3.0	0.10 ± 0.09	0.4 ± 0.1	

[a] PEC, peritoneal exudate cells represented as percentage of human CD15/CD56 positive cells.
[b] *P* comparing tumor weights from mice treated with monocytes + BsMol to those with monocytes + H22.
[c] One mouse died before day 28.

BN/GRP peptide, a method requiring labor-intensive and time-limiting purification of intermediate products twice by high-performance liquid chromatography. Because the COOH terminal of BN/GRP is the active binding site, we hypothesize that manipulating the NH_2 terminus may not interfere with binding and functioning of BN/GRP. We designed a BN/GRP antagonist, adding a cysteine residue to the NH_2 terminus of the peptide (D-Phe[6], Leu-NHEt[13], and des-Met[14]) BN(6–14). The cysteine provides a sulfhydryl group for chemical conjugation. This BN/GRP antagonist with cysteine residue on the NH_2 terminus maintains its functions by inhibiting the growth stimulatory effect of BN on SCLC cells. The new construction is a simple two-step procedure without high-performance liquid chromatography purification. The synthetic BsMol, H22xAntag 2, binds specifically to BN/GRP-R on SCLC cells. On Western blot analysis, the BsMol stains a single band of protein consistent with the molecular weight of BN/GRP-R. These data support our hypothesis that the addition of a cysteine residue at the NH_2 terminus of the BN/GRP antagonist does not alter its biological functions. The new method simplifies the chemical conjugation process.

Cisplatin and etoposide both are the most effective and commonly used chemotherapeutic drugs against SCLC. Paclitaxel is also an active chemotherapy agent to treat SCLC. As a single agent, the overall response to paclitaxel was 53–68% (34). Phase II studies combining paclitaxel with the platinum and etoposide regimen reported response rates of 71–100% in both limited-stage and extensive-stage SCLC (34–36). *In vitro* data suggest that binding of BN/GRP-R increased expression of epidermal growth factor receptor in SCLC cells; the BN/GRP-R antagonist inhibited tumor growth by down-regulating epidermal growth factor receptor (37–38). Because chemotherapy and immunotherapy act through different mechanisms, such a combination is likely to increase tumor cell killing and overcome resistance to chemotherapy.

Targeting BN/GRP-R is an attractive treatment option for SCLC. Although BN/GRP-R is expressed on normal tissues and is involved in a number of physiological functions, the administration of a BN/GRP-R antagonist had only minimal adverse effects in animals (5). Our new approach of immunotherapy targeting FcγRI and recruiting immune effector cells, including monocytes, macrophages, and activated neutrophils, has potential clinical application. Additional studies of this BsMol in the treatment of SCLC are warranted.

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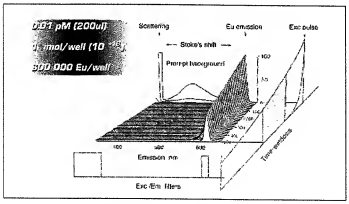
DELFIA Ligands Guide

Perkin precisely.

The DELFIA® ligand family consists of Europium-labeled peptides and proteins designed to be used in ligand-receptor binding assays. DELFIA is based on time-resolved fluorescence lanthanide chemistry, known to be very sensitive and able to detect as low as 1 attomoles of Europium-labeled compound per well. This makes DELFIA a powerful, non-radioactive alternative for demanding ligand-receptor binding studies.



DELFIA technology is based on the use of lanthanide chelate labels with unique fluorescent properties. The fluorescence lifetime of the special signal is several orders of magnitude longer than the non-specific background. This enables the label to be measured at a time when the background has already decayed. The large difference between excitation and emission wavelengths, and the narrow emission peak contribute to increasing the signal to background ratio. The sensitivity is furthermore increased because of the dissociation enhanced principle: the lanthanide chelate is dissociated into a new highly fluoresecent chelate inside a protective micelle.

DELFIA Ligands for G-Protein Coupled Receptors

DELFIA ligands are peptides and proteins labeled with DELFIA europium-N1 chelate coupled to the amino end and/or lysine groups of the protein with the exception of motilin and NDP-αMSH to which the chelate is coupled to carboxyl end of the peptide.

Cytokines	Tachycines	Growth Factors	Others
IL-2	Substance P	EGF	Motilin
IL-4	Neurokinin A		Galanin
IL-5			Neurotensin
IL-8			Bombesin
TNFα			NDP-αMSH

The DELFIA ligand family is constantly growing, so please contact your local sales representative or visit us on the web at www.perkinelmer.com for any updates. For custom labeling, please email labellingservices@perkinelmer.com.

Features	Benefits
➤ High sensitivity	Allows use of recombinant and endogeneous receptor membranes; even with low expression levels
➤ Non-radioactive	No radioactive waste
➤ Long shelf life	Allows flexibility in daily planning and budgeting
➤ Heterogeneous assay	The purity of the membrane preparation is not as crucial as in homogeneous assays
➤ Membrane and whole cell assays both in 96- and 384-well formats	Increased flexibility and throughput
➤ Multiplexing capability	Provides more information at lower cost

Stability

DELFIA ligands are supplied in a lyophilized form and are stable for at least 1 year at 4°C. The exceptions to this are IL-2, IL-4, IL-5 and TNF-α, which are supplied in liquid form. Reconstituted DELFIA ligands are stable for at least one month at -20°C and at least 5 days at +4°C.

DELFIA Ligand-Receptor Binding Assay Formats

DELFIA ligands can be used in several assay formats:

- As a filtration assay using AcroWell™ Filter Plate
- As a solid phase assay using streptavidin coated microtitration plates and biotinylated WGA
- As adherent cells on a cell culture plates (i.e Isoplates)

During the development phase, the biological activity of the Eu-labeled ligand has been demonstrated on AcroWell filtration based assays, with the exception of IL-2, IL-4, IL-5 and TNF-α. The streptavidin coated microplate format places more stringent demands on the receptor expression level. As a rule, the B_{max} should be >0.5 pmol/mg of protein when considering the streptavidin coated assay format. When using ≤ 0.5 pmol/mg, the filtration format is recommended. With AcroWell Filter Plates, up to 20 µg of protein can usually be used without clogging the plate filter. With adherent cells, we recommend to shake the plates to prevent the clogging of the filter. Optimizing the number of cells per well is recommended as well as using a gentle washing step to improve the variation of the assay.

Assay Buffer

DELFIA L*R binding buffer concentrate (cat # CR134-250) is optimized to be used with AcroWell filtration assays:

- 50 mmol/L TRIS-HCl, pH 7.5
- 5 mmol/L $MgCl_2$
- 25 µmol/L EDTA
- 0.2 % BSA

DELFIA L*R binding buffer concentrate:

- Prevents ligands from adsorbing to the well matrix, providing low assay background
- Prevents trace amounts of chelating agents from competing with the Europium ion, providing maximum thermal stability of the assay
- Available at 10x concentrate to allow the addition of necessary buffer additives to each receptor, e.g. protease inhibitors
- Contains Mg^{2+} as divalent cation which is suitable for most receptors

Please note that each receptor may have a slightly different optimum buffer composition. Examples of optimized buffers for filtration assays are given in table 1.

DELFIA Assay Buffer (cat # 1244-106, 1244-111 and 4002-0010) can be used for soluble receptor assays on coated plates, however, it is not recommended to be used in filtration based assays because it:

- Contains inert red dye which may cause clogging in the filtration step
- Contains Tween 40 which harms cells and membranes
- Doesn't contain any divalent cation which is necessary for many ligand binding assays

Wash Buffer

The washing step should allow efficient removal of any free Eu-label without disturbing the ligand-receptor complex.

DELFIA L*R wash concentrate (cat # CR135-250) contains TRIS-buffer supplemented with $MgCl_2$ which is used in many ligand binding assays. It can be used in any DELFIA ligand-receptor binding assay.

DELFIA Wash Concentration (cat # 1244-114 and 4010-0010) can be used in soluble receptor assays on coated plates.

DELFIA Filtration Assay Protocol

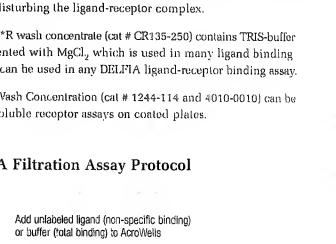


Figure 1. Schematic protocol for DELFIA ligand-receptor filtration assay displacement curve. Assay volumes shown in brackets are recommended to be used with AcroPrep™ 384 Filter Plate (Pall Life Sciences). This protocol has shown to be optimum for the DELFIA ligands and receptors shown in Table 1, except for NDP-αMSH which should be incubated 120 minutes at 35 °C.

Table 1. Examples of K_d and K_i values and signal-to-background (S/B) ratios received with the indicated receptor membrane preparation when using AcroWell 96-well filtration plates and buffers shown in the table. Similar K_i and K_d values have been obtained with AcroPrep 384 filtration plates (data not shown)

Eu-ligand	Receptor	Receptor source	B_{max} (pmol/mg) of protein	µg/well of membrane protein	fmol/well of receptor	K_d (nmol/L)	K_i $(nmol/L)^{-1}$	S/B	Buffer additives added to DELFIA L*R binding buffer	DELFIA L*R wash buffer
Motilin	recomb. rMot	RBHMOT6 expressed in HEK-293 cells (PerkinElmer)	18.4	0.5	9.2	0.4	0.5	11	1 % PVA (Polyvinyl alcohol, MW 6000)	yes
Interleukin-8	recomb. hCXCR2	RB-CX2M expressed in HEK 293 EBNA cells (PerkinElmer)	1.7	3	5.1	1.9	0.5	8	2 mmol/L CaCl2, 50 µg/mL saponin	yes
Galanin	recomb. hGalR2	RB-G_2M expressed in CHO cells (PerkinElmer)	3-6	1	3-6	2.6	2.4	15	0.2 % Bacitracin, 1 mmol/L phenylmethylsulfonyl fluoride	yes
* EGF	endog. hEGFR	RB-EG2M expressed in A431 cells (PerkinElmer)	11	1	11	0.8	0.4	12	130 mmol/L NaCl, 50 µg/mL saponin	yes
Neurotensin	recomb. hNT1	RB2NTM expressed in HEK-293 cells (PerkinElmer)	1.06	5.7	6	1.1	0.3	7	none	yes
Neurokinin A	recomb. hNK2	S1102/10 expressed in CHO cells (Amersham)	2.8	1	2.8	1.7	0.4	13	1 mmol/L phenylmethylsulfonyl fluoride	yes
Substance P	endog. rPAT	S1102/51 expressed in IC11 cells (Amersham)	0.25	12	3	0.6	0.5	7	0.05% Tween-20	yes
NDP-αMSH	recomb. hMC3 recomb. hMC4 recomb. hMC5	RB2MC3S4 RB4MC3M4 RB4MC3M expressed in HEK-293 cells (PerkinElmer)	1.95 1.63 12.2	1 2 0.5	1.95 3.26 6.1	0.45 0.6 1.1	0.13 0.27 0.22	14 15 15	1 mmol/L phenylmethylsulfonyl fluoride and 5 mmol/L CaCl2	yes yes yes
Bombesin	[illegible]	[illegible]BN4 expressed in CHO cells (PerkinElmer)	3.4	0.4	1.36	0.5	0.24	4	50 µg/mL saponin	yes
	[illegible]	RB2M expressed in HEK293 cells (PerkinElmer)	9.3	0.2	1.86	0.5	0.2	8		yes

[1] Eu-ligand concentration is close to the K_d value in displacement studies.

Table 2. DELFIA ligand-receptor assay comparison to radioactive filtration assay.

ligand	K_d (nmol/L) DELFIA assay	K_i $(nmol/L)^{-1}$ DELFIA assay	K_i $(nmol/L)^{-1}$ rad assay	K_d (nmol/L) rad assay
* Motilin	0.4	0.5	1.5	0.24
Interleukin-8	1.9	0.5	0.19	0.29
Galanin	2.6	2.4	–	0.2 – 0.3
EGF	0.8	0.4	5.8	0.8
Neurotensin	1.1	0.3	0.25	0.23
Neurokinin A	1.7	0.4	2.2	0.6 – 0.9
Substance P	0.6	0.5	0.02-0.5	0.2
NDP-αMSH MC3 MC4 MC5	0.45 0.6 – 1.1 1.1	0.13 0.27 0.22	0.44 1.0 3.8	0.22 0.15 1.5
Bombesin BB1 BB2	0.5 0.5	0.2 0.24	0.80 0.17	0.047 0.037

Multiplexing Capability

The unique chemical properties of lanthanides allow you to measure up to four ligand-receptor binding events within the same well. High signal to background value is achieved by using both temporal and spectral resolution. When optimizing the multilabel assay, please contact technical support for more detailed information (in Europe: techsupport.europe@perkinelmer.com, in the U.S. and the rest of the world: techsupport@perkinelmer.com). To get Samarium (Sm) and Terbium (Tb) labeled ligands, please email labellingservices@perkinelmer.com.

Products

Product code	Product	Package
AD0208	DELFIA Eu-labeled motilin	60 pmol* (enough for approximately 960 wells)
AD0209	DELFIA Eu-labeled motilin	240 pmol* (enough for approximately 4800 wells)
AD0213	DELFIA Eu-labeled interleukin-8	160 pmol* (enough for approximately 960 wells)
AD0214	DELFIA Eu-labeled interleukin-8	700 pmol* (enough for approximately 4800 wells)
AD0215	DELFIA Eu-labeled galanin	200 pmol* (enough for approximately 960 wells)
AD0216	DELFIA Eu-labeled galanin	850 pmol* (enough for approximately 4800 wells)
AD0217	DELFIA Eu-labeled EGF	350 pmol* (enough for approximately 960 wells)
AD0218	DELFIA Eu-labeled EGF	1400 pmol* (enough for approximately 4800 wells)
AD0219	DELFIA Eu-labeled neurotensin	200 pmol* (enough for approximately 960 wells)
AD0220	DELFIA Eu-labeled neurotensin	750 pmol* (enough for approximately 4800 wells)
AD0221	DELFIA Eu-labeled neurokinin A	300 pmol* (enough for approximately 960 wells)
AD0222	DELFIA Eu-labeled neurokinin A	1200 pmol* (enough for approximately 4800 wells)
AD0223	DELFIA Eu-labeled substance P	200 pmol* (enough for approximately 960 wells)
AD0224	DELFIA Eu-labeled substance P	800 pmol* (enough for approximately 4800 wells)
AD0225	DELFIA Eu-labeled NDP-αMSH	200 pmol* (enough for approximately 960 wells)
AD0226	DELFIA Eu-labeled NDP-αMSH	800 pmol* (enough for approximately 4800 wells)
AD0227	DELFIA Eu-labeled bombesin	150 pmol* (enough for approximately 960 wells)
AD0228	DELFIA Eu-labeled bombesin	600 pmol* (enough for approximately 4800 wells)
CR400-600	DELFIA Eu-labeled TNFα	600 pmol
CR401-650	DELFIA Eu-labeled interleukin-2	650 pmol
CR402-400	DELFIA Eu-labeled interleukin-5	400 pmol
CR403-060	DELFIA Eu-labeled IL-4	60 pmol
1244-104	DELFIA Enhancement Solution	50 ml
1244-105	DELFIA Enhancement Solution	250 ml
4001-0010	DELFIA Enhancement Solution	1000 ml
CR134-250	DELFIA L*R binding buffer concentrate (10x)	250 ml
CR135-250	DELFIA L*R wash concentrate (25x)	250 ml
AAAND-0005	DELFIA Streptavidin-coated yellow plate, 96 well	10 plts
RBHMOTM	Human motilin receptor	400 UA
RBHCX2M	Human recombinant interleukin-8b CSCR2 receptor	400 UA
RBHEGFM	Human endogenous epidermal growth factor receptor	400 UA
RBXNT1M	Human recombinant neurotensin receptor subtype 1	400 UA
RBXMC3M	Human recombinant melanocortin receptorMC3	400 UA
RBHMC4M	Human recombinant melanocortin receptor MC4	400 UA
RBXMC5M	Human recombinant melanocortin receptor MC5	400 UA
RBHBS1M	Human recombinant bombesin receptor subtype 1	400 UA
RBHBS2M	Human recombinant bombesin receptor subtype 2	400 UA
6110551 (Amersham)	Human endogenous neurokinin receptor subtype 1	200 UA
6110510 (Amersham)	Human recombinant neurokinin receptor subtype 2	200 UA
5020	AcroWell Filter Plate, 96 well	10 plts
1450-584S	B&W Isoplate-96 TC	2 plts
1450-517S	Isoplate-96 TC	2 plts

* The number of the wells varies depending on the assay conditions.

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